ASPECTS OF THE ECOLOGY OF SALMONELLAS IN POULTRY LITTER

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DECLARATION

I hereby declare that the whole of the work submitted under the title "Aspects of the Ecology of Salmonellas in Poultry Litter" as a thesis for the degree of "Doctor of Philosophy" of the University of Edinburgh was composed by myself and is the result of an original investigation undertaken by myself. All authors and works consulted are fully acknowledged and the material published prior to submission is appended.

S.C. Morgan-Jones.

S.C. Morgan-Jones.

ABSTRACT

The history of poultry production, the current conditions of broiler rearing and possible future trends are discussed in relation to the industry as a source of salmonella infection for man. The survey of literature showed that knowledge of the occurrence of salmonellas in broiler houses is fragmented and confused but it does indicate that some litters are inhibitory to salmonellas. Elucidation of the mechanism of inhibition could suggest methods which would possibly limit infection of chicks and the contamination of the natural evironment by contaminated litter.

Using a method for enumerating salmonellas developed for this investigation this attribute of litter was confirmed and shown to be a combination of physico-chemical parameters together with bacterial antagonism. The wood shavings used as basal material are inert, the faeces provide the active ingredients.

The interaction between salmonellas and litter was found to be in three phases.

Phase 1 (0 to 4-7d)

Salmonellas are inhibited by formaldehyde adsorbed on the wood shavings during fumigation. This chemical is eliminated in many ways including dissolving in the aqueous phase, volatilization, reaction with faecal material and degradation by bacteria.

Phase 2 (4-7 to 25-35d)

During this period salmonellas may multiply in these litters although the physico-chemical parameters are altering so that in:-

Phase 3 (25-35 to slaughter)

the salmonellas are inhibited. The degree of inhibition is directly related to the rise in pH level. A water activity of 0.4-0.6 units also contributed to the inhibition, above this level salmonellas multiply and below survived. Volatile fatty acids did not contribute to the inhibitory effect at alkaline pH's whereas the gaseous environment of litter and temperature appear to favour the multiplication of salmonellas. Bacterial antagonism was shown to be due both to direct inhibition and indirectly by degradation of uric acid to ammonia with a consequent rise in pH value.

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It would not be possible to carry out this programme without the co-operation of the management and staff of the poultry industry and I would like to use this opportunity to thank them for all the support which they have afforded to me in all my work with poultry.

Finally, I am very grateful for the forbearance of my Mother

and Sister and in all the practical ways in which they have helped me to complete this investigation.

ABBREVIATIONS AND DEFINITIONS OF TERMS

Abbreviations

A_w - Water Activity

I - Inhibition

MC - Moisture content

MPN - Most Probable Number

NA - Not available

NT - Not tested

PI - Partial inhibition

RH - Relative Humidity

VFA - Volatile Fatty Acid

Statistical Terms

Analysis of Variance

$$F - \text{value} = \frac{\text{Mean Square due to treatments}}{\text{Mean Square due to error}}$$

df. = degrees of freedom

Analysis of Regression

R^2 = Coefficient of Correlation

* = Significant at P = 0.05

** = Significant at P = 0.01

*** = Significant at P = 0.005

Definition of Terms relating to death and survival

1. Growth in solution

The presence or absence of growth as determined by the presence or absence of turbidity as detected visually when illuminated by a

good light.

2. Non-inhibited cells (named growth)

Cells capable of growing in suitable non-selective and selective media at an optimal temperature in the time customary for the bacterial species cultured.

3. Inhibited, undamaged cells

No visible growth in solutions under test, but when an aliquot is transferred to an appropriate selective medium cells show growth within the time prescribed for the medium.

4. Inhibited physiologically damaged cells

No visible growth in solution under test or in selection media on transfer, but growth in non-selective media at optimal temperature.

5. Death

No visible growth in solution under test or in appropriate non-selective media after 3d at optimal temperature.

EXPRESSION OF MICRO-ORGANISMS - LOG $_{10}$ CONVERSION

A. INTRODUCTION

1. Food poisoning may result from the consumption of food with pathogenic bacteria. In man, the symptoms of this condition which are mainly gastro-intestinal are distressing to the patient often resulting in loss of working time and in the case of the young or elderly may be fatal.

The salmonella group of bacteria have been implicated in many outbreaks of food poisoning; cows milk and poultry meat being the sources most often implicated. In Scotland the cessation of the granting of licences for the sale of raw milk after August 1983 so effectively introducing the compulsory heat treatment of milk, (Statutory Instrument, 1980), will eliminate this source of infection. In contrast the possibility of erradicating salmonellas from the broiler flock would not appear to be either practical or economically feasible in the near future. Therefore the poultry industry should attempt to reduce the level of infection at those points where controls could be effective, the main places being the nest box material, the handling of the eggs on the breeder-rearing farms, the period the birds are in the broiler house and at slaughter. The control of infection in eggs has been studied for many years, but relatively little work has been carried out on husbandry practice which may affect the survival of pathogenic bacteria in poultry units.

The following literature review describes the development of the broiler industry, our current knowledge of the distribution and

multiplication of salmonellas within poultry units and the distribution chain to the consumer. Assessment of such information is a pre-requisite to appreciating those areas which have the greatest influence on the spread of these bacteria. Once these foci have been established it would be possible to suggest modification in husbandry techniques which would aid in reducing the level of infection in the flocks.

B. THE POULTRY INDUSTRY IN GREAT BRITAIN

1. The Historical Background of the Poultry Industry

Sculptures of geese in the Pyramid of Sah'ara in Egypt built in 4000 BC are the first recorded evidence of poultry husbandry although the hen (Gallus domesticus) has been domesticated since time in memoria. The earliest description of fattening chickens is that of Pliny who described a method of force-feeding (cramming) practiced by citizens of Delos in Ancient Greece. The Romans are credited with bringing the chicken to Great Britain in 55 AD but as the Druids forbade the use of chickens for meat these birds were mainly bred for cockfighting.

In the documents of the Middle Ages there are many descriptions of all aspects of cockfighting, a very popular sport of the time, but little advice on the fattening of poultry. However, poultry must have been raised for meat on a domestic scale as chickens are listed in the menus for medieval banquets and Gasgoigne (1340-1410) tells of tenants paying their quarterly rent at Midsummer with fowl.

By 1808 in England, Wokingham had become a centre for the rearing of poultry which were fattened by cramming, later the practice became widespread in Sussex and Surrey. The "fatteners" employed collectors of "higglers" to purchase birds from the rearers. Birds to be sold as "half-fat" were kept in outside cages for 7 to 10 days while those to be sold as "fat" were similarly housed in barns. All these birds were 3 to 4 months old at slaughter and so

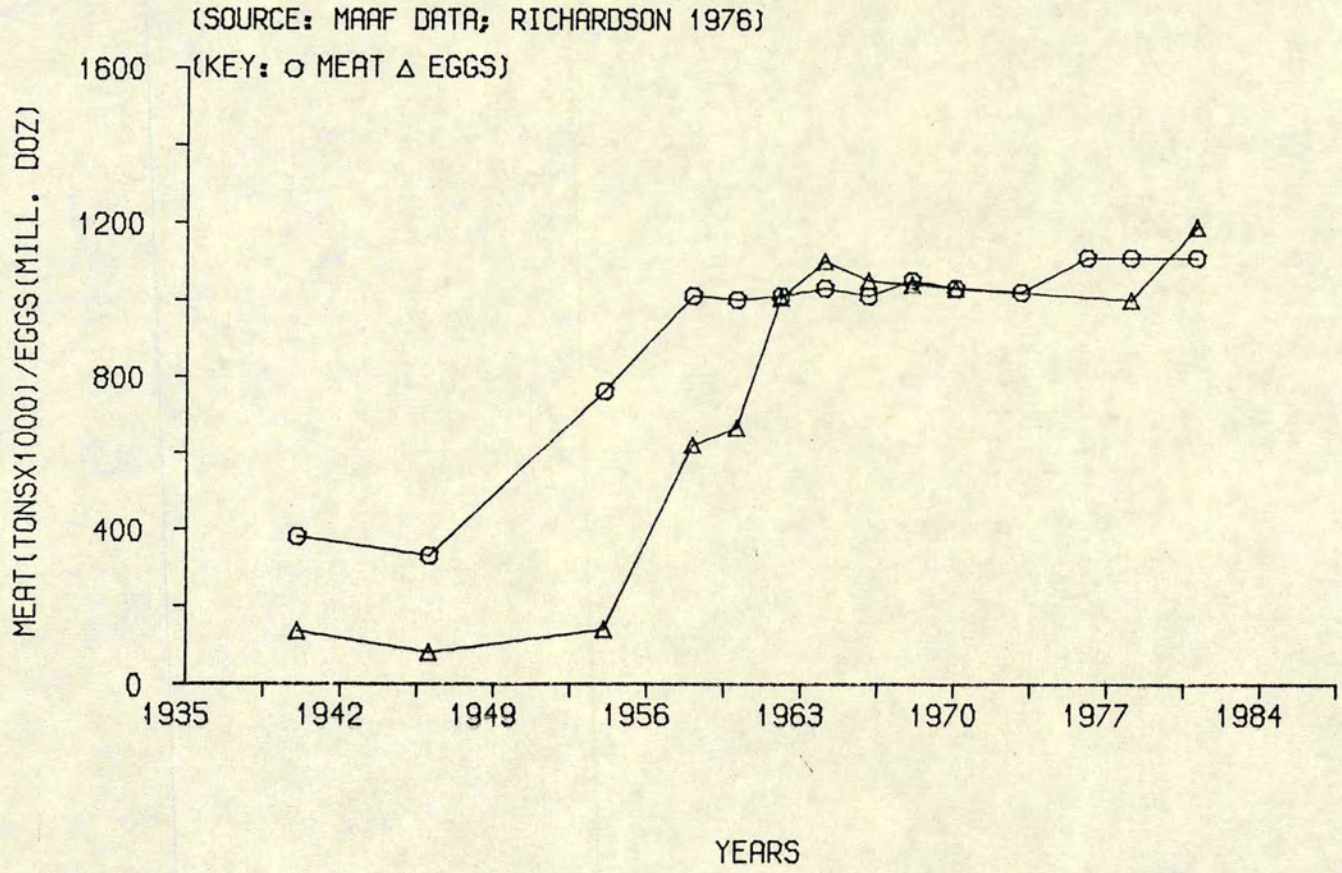
were only suitable for roasting or boiling. Relatively large numbers of birds were produced in this way for it is recorded that a "Mr. C. Brooks" sent 300,000 birds to the London markets in 1898.

In all other areas of the country poultry rearing was mainly the province of the farmer's wife who kept hens for their eggs. Therefore the only poultry meat available was the carcase of these birds when they were slaughtered at the end of their laying period.

At this time in America it was common to rear birds for slaughter at 8 to 12 weeks when 0.75-1 kilo in weight, as these were suitable for broiling i.e. cooking over a fire on a gridiron, these were termed broilers (Wingfield & Johnson, 1857; Lever, 1910; Brown, 1930).

The poultry industry continued in its traditional form until the end of the first World War when some service men on demobilisation were attracted to country life; in this context poultry keeping afforded a lucrative means of attaining this lifestyle. As a result poultry enterprises were established on small acreages, the birds being kept in folds and straw-yards primarily for egg production. Despite the concentration on egg production at this time 70 per cent of the eggs consumed were imported into Great Britain (Anon, 1935a). Lack of experience, capital and high mortality of the birds led to so many financial failures that in 1935 the Ministry of Agriculture set up a commission to suggest improvements in the methods of poultry husbandry (Anon, 1935b), however, the onset of World War II prevented these recommendations being implemented.

FIG 1.1 TRENDS IN THE PRODUCTION OF CHICKEN MEAT AND EGGS IN GREAT BRITAIN FROM 1935 TO 1980.



At that time in the United States this sector of the industry was expanding rapidly as can be seen from the figures for California, where in 1935, 1 million birds were reared for meat, by 1939 the number was 5.8 million and by 1944 the numbers had risen to 15.9 million (Asmundson & Lerver, 1951). This rapid development stimulated a group of representatives of the scientific and commercial sector of the British poultry industry to tour North America in 1946 to study these intensive methods (Anon, 1947). The visitors enthusiastically reported that the majority of broiler birds were housed in deep litter systems at the density of 0.4 to 1.2 m^2 per bird, fed by a cafeteria system and that some of the houses were ventilated by fans. They were also surprised that one man could be responsible for as many as 20,000 birds but reported that high mortality rates were the greatest limitation to the system.

When the war-time restrictions on animal feedstuffs was relaxed the emphasis in the poultry industry continued to be on egg production (Figure 1.1) so that by 1970 Great Britain had become 99 per cent self-sufficient in this commodity. By contrast the growth of the poultry meat market was slower but by 1960 sufficiently high numbers of broilers were being reared that broilers were listed as a separate item in the official statistics of the United Kingdom (Anon, 1961). About this time it was also recognised that poultry production was moving from a farm-based industry to a more factory-type system (Cole, 1954; Thornber, 1960), and the efficient marketing of this product was recognised as crucial to the success of this system.

In the last two decades a great deal of research has been expended in the development of the controlled environment house in use today, improvement of nutrition and the breeding of suitable strains of birds to satisfy the requirements of the expanding meat market.

2. The Contemporary Poultry Industry

(a) The structure of the industry

In Great Britain about 400 million broiler birds are reared per year in large units by specialised producers, 73 per cent of all birds being produced by the "top ten" producers (Anon, 1981). The economics of the poultry industry is based on the production of a large number of birds with a low profit margin per bird (Anon, 1976) so that the white meats now compete favourably with red meats mainly on the basis of price. A recent report has shown that in 1982 in Great Britain the sale of chicken meat was 369,000 tons, an increase of 9 per cent in sales over 1981 while 398,000 tons of beef were sold representing a decrease of 5 per cent. If this trend continues chicken meat sales could overtake the sale of beef by the end of the 1980's (Anon, 1983). In part the increased sales of white meat are due to the popularity of the trend of dividing the carcasses into portions which are sold either raw or cooked. The success of this approach together with attractive advertising had led to many retailers sharing the experience of Marks & Spencers PLC where chickens are their most successful item in terms of money taken at the cash tills (Davies & Herrmann, 1981).

The majority of modern enterprises producing broilers are vertically integrated units the feed mills, breeding stock hatchery, broiler rearing flocks, slaughter and processing facilities being owned or controlled by one company.

(b) Housing of the broiler

The typical broiler house has an area of approximately 2000 m^2 housing up to 30,000 birds in either one or two rooms. Most producers aim to raise 4 to 5 flocks each year per house. There are a great variety of house designs depending on the builder, site and personal preference. The majority of houses are either timber lined with plywood, asbestos sheets, faced by brick, or concrete blocks, the minimum height of the walls being 1.5m. The roof is either a double skin of asbestos sheet lined and filled with compressed straw or insulated board covered with roof felt. Floors are typically concrete, on a damp proof membrane, sloping slightly towards the side walls (Anon, 1981). The pelleted feed is normally moved by gravity into an automatic distribution system either to static troughs or onto a moving chain belt. Chick crumbs are fed for the first 21 to 28d when the ration is gradually replaced with a rearer's formulation (Anon, 1980). Houses are usualy lit for 23 h/d the light density depending on the chosen rearing programme. The heating is invariably by gas heaters suspended from the roof beams along the centre of the house, the temperature being maintained, initially at 29 to 32°C then reduced to 21°C by 2.5 to 3 weeks. All modern houses have a forced ventilation system, a great variety

of designs being advocated (Anon, 1976a). A fixed stocking rate of 0.05 to 0.09 m^2/birds is normal, the birds remaining in the house until slaughter at 45 to 56d (Anon, 1980) with a liveweight of 1.1 to 1.6kg.

Some stockmen favour the brooder system of rearing young chicks, the chicks being restricted to an area below the heaters, the area being extended daily until the chicks have access to the whole house by the tenth day. Other stockmen prefer to allow chicks access to the whole house immediately but the birds do tend to congregate under the heaters where the food and water are tactically placed.

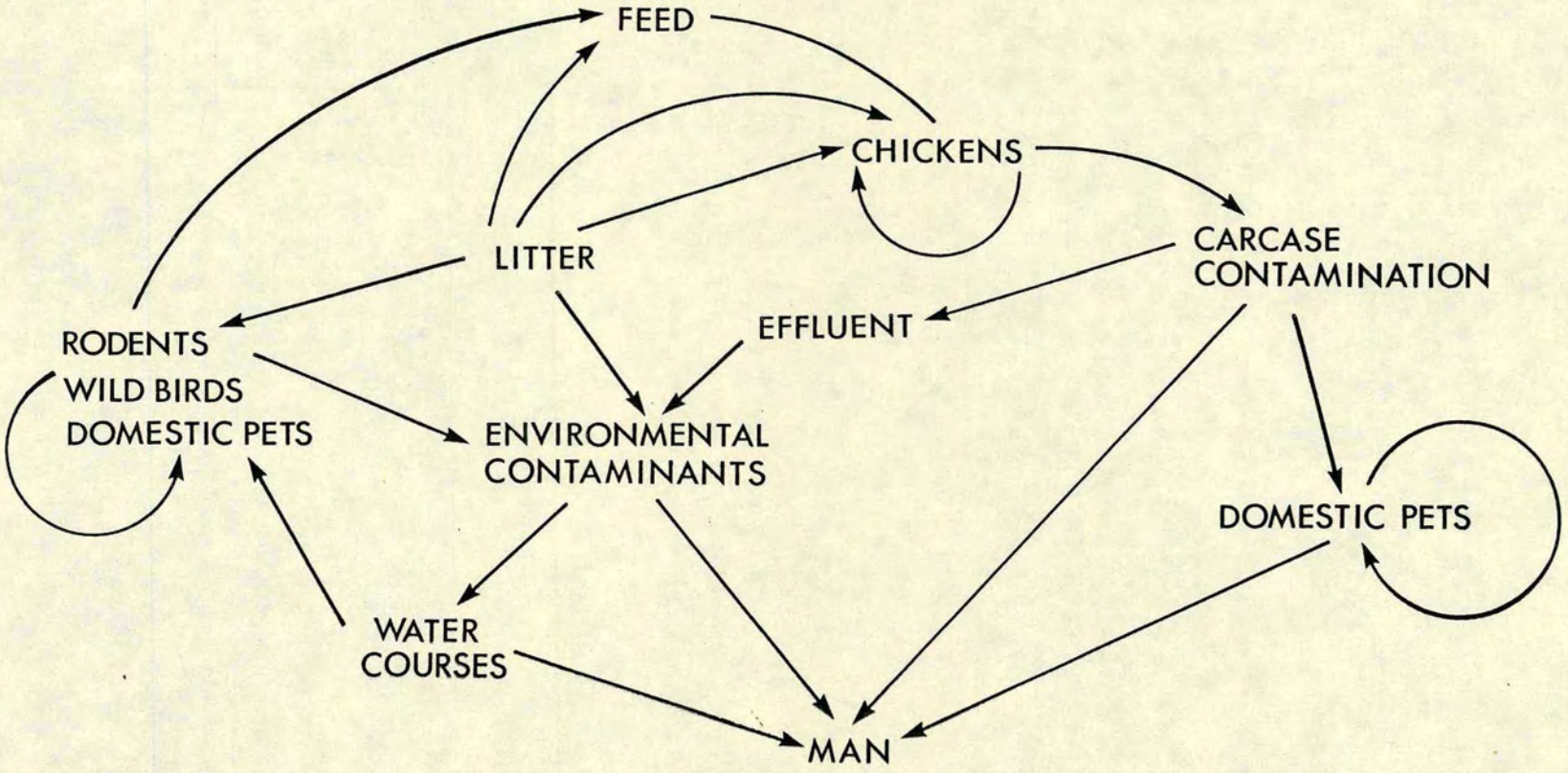
(c) The cleaning and disinfection of poultry houses

After each batch of birds are removed the house is sprayed with water containing detergent to lay the dust and the equipment dismantled to be cleaned separately. The litter is removed by a tractor with a foreloader unit. The building is then disinfected using a high pressure, low volume power hose, or steam jet, while small items are hand-brushed and soaked in a disinfectant solution (Anon, 1978a; 1982).

Ideally 48 hours before the day-old chicks are to be placed in the house, the building and contents are fumigated either by heating paraformaldehyde (100g per 30m^2) in electrically heated pans designed for the purpose or by spraying an aerosol of formalin into the atmosphere (28ml per 6m^2) (Anon, 1978b; 1982). A few hours before the chicks are placed the house is ventilated. The cleaning procedure

FIG.1.2

THE POSSIBLE PATHWAYS OF SALMONELLA INFECTION BETWEEN POULTRY AND MAN



normally takes 20 days to complete.

(d) The utilization of poultry litter

The utilization of the litter presents a problem for most enterprises. Although many poultry farmers are able to spread some of their litter as fertiliser either on their own ground or by arrangement with a neighbouring farmer, a great deal of this material is deposited in quarries, disused railway lines, etc. If the whole bulk of the litter does not heat up to 52°C there is a possibility that pathogens may still be present. These pathogens can contaminate the natural environment especially if a litter is stored as a pile in a field where these bacteria will enter the water courses in the effluent or are distributed on the feet, beaks or feathers of wild birds (Kraft et al., 1969). The possible pathways by which livestock and man could become infected are shown in Figure 1.2.

Poultry litter can also be used as an ingredient for stock after treatment to ensure that the material is free of disease organisms. Many methods are used for treating poultry litter on the farm, ensilage being the most commonly used system (Syrett, 1977). This may be carried out by either stacking the litter on a concrete floor, in towers or in polythene bags. Of these the first method is the cheapest and so the most attractive system from the economic point of view. Additives such as maize forage (Harmón et al., 1975), mollasses, formic acid, propionic acid (Syrett, 1977) and grass (Morrison pers. comm.) are sometimes included to increase the

nutritive value of the resultant feed. The ensilage process is completed when the material has heated up and fermented to an acidic pH.

On a commercial scale the litter is steam heated to 120°C for 30 mins and then dried before being incorporated as a protein supplement in many proprietary feed rations.

A wide range of livestock have been successfully fed treated poultry litter including dairy cows (Kneale & Garstang, 1975), beef cattle (Muller & Strosova, 1968; El-Sabban et al., 1970; Webb & Fontenot, 1975) sheep (Flipot et al., 1975), pigs (Geri, 1968; Müller & Strosova, 1968) and even poultry (Conni, 1969; Hennig et al., 1975; Vuori & Nasi, 1977).

Although at present this use of poultry litter is not popular in the near future economic pressures will probably result in an increase in the use of ensiled litter in stockfeed especially for cattle feed.

3. The Poultry Industry of the Future

The emergence of a very vocal, persuasive animal welfare lobby coupled with the greater interest by consumer groups in the method of rearing all farm animals has led to widespread criticism of some systems of animal production. In particular the confinement of chickens in cages has been condemned. This system is mainly restricted to egg production but there are advantages in rearing broiler birds in cages, for instance cages enable a higher density of birds to be housed per unit space so increasing the number of

birds managed per man in more agreeable working conditions. As the cages could also be used to transport the birds to the slaughter lines the birds are less stressed prior to killing resulting in a carcass of higher organoleptic quality (Scholtyssok, 1980). However, public aversion to this management system coupled with the high capital cost of the equipment would appear to make it likely that in the near future all broilers continue to be reared on litter.

It is also probable that in the future deep litter systems will be increasingly used for birds kept for egg production as the data collected by the animal welfare campaigners suggest that producing eggs by less intensive methods is an economic proposition. These groups consider that the housewife will be willing to pay the 10 per cent rise in price resulting from the changes in rearing systems (Cornell, 1981).

While the question of the economics of egg production, the arguments on egg washing and staff problems are outwith the scope of this apercu it would appear there will be a movement from caged systems to litter systems in the future. Therefore it is essential that the emphasis in research and development of poultry rearing systems should be concentrated on deep litter systems rather than caged systems.

C. THE IMPORTANCE OF SALMONELLAS WITHIN THE POULTRY INDUSTRY

1. Poultry Products in the Human Diet in Relation to Human Health

Improvements in the handling of eggs at the farm and more rapid distribution to the retail shops have ensured that these products now reach the consumer both fresher and in a more hygenic condition than ever before. As a consequence shell eggs are not significantly contaminated with disease producing bacteria therefore there are now virtually no cases of human infection resulting from the consumption of eggs a situation very different from the past. Those incidences of human bacterial food poisoning when eggs have been implicated result from the use of bulk liquid egg melange or imported dried egg products. Today the Regulations (Statutory Instrument, 1963) detailing the heat treatment and storage of these products are strictly adhered to, so that these sources of bacterial food poisoning are now largely of academic interest (Gilbert & Hobbs, 1980). In contrast the possibility of bacterial food poisoning derived directly or indirectly from poultry meat is a much discussed emotive subject which tarnishes the image of the industry.

Statistics collected by the Communicable Disease Centres in Great Britain show that the numbers of outbreaks of salmonella bacteria food poisoning, where poultry meat has been implicated, have increased in recent years. There are a number of factors which could be responsible for this occurrence, but most importantly the extensive consumption of poultry meat, especially in the catering industry, and the greater efficiency in investigating outbreaks of food poisoning both in the field and the

laboratory. In addition there is an increasingly wider range of serotypes of salmonellas implicated as potential pathogens.

The most publicised incidents of salmonella food poisoning have involved food from catering premises. While correct handling should prevent the spread of salmonellas within the kitchens and thorough cooking will render food salmonella-free, unfortunately the education of all food handlers is a formidable or impossible task (Anon, 1977a). Therefore there is increasing public pressure to implement programmes to eliminate salmonellas from the poultry stock so that all carcasses would be guaranteed free from salmonellas on receipt at the kitchens. In Canada the cost of a possible plan to eradicate salmonellas from their National flock was estimated, at 1977 prices, to be $67 million per year. This represents a $1 benefit for each $13 expended and so therefore not economically feasible (Finn and Mehr, 1977). However, a better understanding of the way in which salmonellas cycle in poultry units may allow modifications in management procedures which would then reduce the level of infection and so provide a less costly alternative to eradication (Derbyshire, 1971).

2. Salmonellas and Broiler Chicken

(a) Clinical cases of avian salmonellosis

Avian salmonellosis or paratyphoid is the term applied to infection of birds by any serotype of the salmonella group except *Salmonella pullorum* and *S. gallinarum*. These serotypes result in conditions termed bacillary white diarrhoea and fowl typhoid

respectively both of which have now been eliminated in Great Britain by an eradication policy (Anon, 1965).

Clincal cases of avian salmonellas are rare in the British flock but when it occurs it is nearly always confined to chicks of less than three weeks of age. Infected birds are drowsy with closed eyes, ruffled feathers, a desire for heat and water, sometimes with diarrhoea and vent pasting. Mortality may be as high as 50 per cent (Gordon & Jordan, 1982; Coutt, 1981). Today when clincial disease occurs the salmonellas involved are normally Salmonella bareilly, S. gallinarum-pullorum, S. infantis and S. typhimurium (Mulder, 1982).

(b) Subclinical infection of the apparently healthy bird

Subclinical infection of chickens presents a greater problem to the poultry industry than clinical cases, as these asymptomatic birds while appearing healthy are able to pass on the infection to other birds in the flock. The Poultry Meat (Hygiene) Regulations (Statutory Instrument, 1976) rely solely on a ante-mortem health inspection prior to slaughter, which normally consists of a visual assessment of the flock coupled with a scrutiny of the health records of the flock. This will not detect the presence of sub-clinical infection, therefore infected birds may be slaughtered and consequently contaminated carcasses are released to the retail market.

In modern intensive management where large numbers of fully susceptible birds are reared together in a closed space there is

potential for almost unlimited multiplication and transfer of infection following the initial introduction of salmonellas into the environment.

The infection level of clinically healthy birds in the national flock has been variously estimated from 0.5 to 14.8 per cent (Gordon, 1970; Elliott & Michener, 1961) although individual batches appear to be salmonella-free even in enterprises known to have a problem with salmonellas (Morgan-Jones, 1977). Unlike clinical incidents of salmonellosis, where one serotype always predominates, in surveys of asymptomatic birds a wide range of serotypes are isolated in a single flock, 20 serotypes being the highest number found to date (Aarsse, 1976).

When subclinically infected birds are slaughtered the salmonellas present in the carcase will act as a source of contamination for the equipment of the factory and so be transferred to other carcasses. Using marker organisms it has been shown that one infected carcase can result in the contamination of up to 200 of the following carcasses on the evisceration line (von Schothorst et al., 1972; Mead et al., 1975) and also introduce this bacteria into the wash and chill tanks (Morgan-Jones, 1977). An infected carcase may remain a hazard to public health for a considerable length of time as the salmonellas will survive in the chilled and frozen carcase which if improperly handled could lead to a food poisoning incident.

It is difficult to assess the trends in human and animal infections as the techniques for laboratory diagnosis are

constantly changing in addition to variations in the reporting systems. The most recent major change in the reporting method has been the introduction of the Zoonoses Order in 1975 (Statutory Instrument, 1975) which transferred the reporting of isolations of salmonellas from a voluntary to compulsory basis. This legislation however, does not specify the sampling programme, so the data collected is dependent on vigilance of the industry and can be inadvertently distorted by a change of policy of a large poultry producer. Therefore the onus continues to rest with the individual companies to reducing the level of infection to a minimal level so safeguarding their reputation.

3. The Occurrence of Salmonellas in Broiler Enterprises

(a) Birds

In the British broiler industry the main foci of salmonella infection are the broiler-breeder flocks. The infection of the eggs can be transovarian, although these eggs rarely hatch; or immediately after laying due to soiling by the faeces-infected nest materials, egg trays, hands of staff etc. While most eggs destined for hatching are fumigated or washed at the farm these procedures are not always correctly carried out and so contaminated eggs may be received at the hatchery (Buckle et al., 1981). Conditions within setters and hatchers are ideal for the multiplication of salmonellas which can result in the infection of the newly hatched chicks (Buxton and Gordon, 1947). The chicks can then introduce these bacteria into a previous salmonella-free house (Morgan-Jones,

1982). The source of infection may be water-drinkers, bedding, feed or even the structure of the house (Barker, 1966).

Birds have been artificially infected by various methods such as percoacal infection (Leaney et al., 1978; Weinach et al., 1979) or intra-peritoneal injection (Milner & Schaffer, 1952) but oral administration is the most commonly used method (Buxton & Gordon, 1947; Botts et al., 1952; Milner & Schaffer, 1951; Brownell et al., 1969; Smith & Tucker, 1975; 1980a & b; Rigby & Pettit, 1979; Weinach et al., 1979). The numbers of bacteria which will initiate infection has not been established with any degree of certainty, the numbers per dose being reported in the literature ranging from as low as one per 2g in feed (Gordon & Tucker, 1965) to 10.0/ml (Brownell et al., 1969). It will probably never be possible to predict the "effective" dose as the level of infection established also depends on other factors such as age (Milner & Schaffer, 1952), stress (Brownell et al., 1969), virulence of serotype (Henderson et al., 1960; Smith & Tucker, 1980b), physiological state of bird and management systems. However, it is generally accepted that an infective dose of over 5.0 bacteria per bird will result in 100 per cent of the birds becoming infected while with a lower dose the numbers of affected birds will vary from 1.9 per cent (Gordon & Tucker, 1965) to 100 per cent of birds depending on the factors already discussed.

When the bird ingests salmonellas from any source the intestine is the first site to become infected. Salmonellas may persist but do not proliferate in the crop (Turnbull & Snoeyenbos, 1974) whilst

it would appear that these bacteria can multiply within the intestine (Shaffer et al., 1957). When the sites within the intestine were studied by Fanelli et al. (1979) they found that the caecum was the most sensitive site for salmonellas and examination of swabs of the caecal contents were the best method of detecting salmonellas in the digestive tract. The organisms then pass via the blood stream to other organs of the body where they remain as a focus of infection within the bird. The translocation of bacteria through the intestinal wall was first demonstrated at Reading by Fuller and Jayne-Williams (1970) but they did not establish the site of translocation. When Turnbull and Snoeyenbos (1974) studied the translocation of salmonellas they found that the major sites of movement in day old chicks were the crop and caeca and it was possible in any part of the intestine.

As already discussed in the broiler industry the main problem is the symptomless bird which while presenting all the features of health carries infection. When the internal organs of "healthy" birds have been examined salmonellas has been recovered from the liver (Knivette, 1971; Kumer et al., 1971; Hensen & Matheld, 1972) spleen (Turnbull & Snoeyenbos, 1974) gall bladder (Buxton & Gordon, 1947; Hensen & Matheld, 1972; Brown et al., 1973) heart (Morgan-Jones, 1980) lungs (Brown et al., 1973).

In some birds the infection may persist in the gut-associated tissues without excretion. If these birds are stressed or the immune response is suppressed such an infection may be reactivated leading to the excretion of the organism in the faeces.

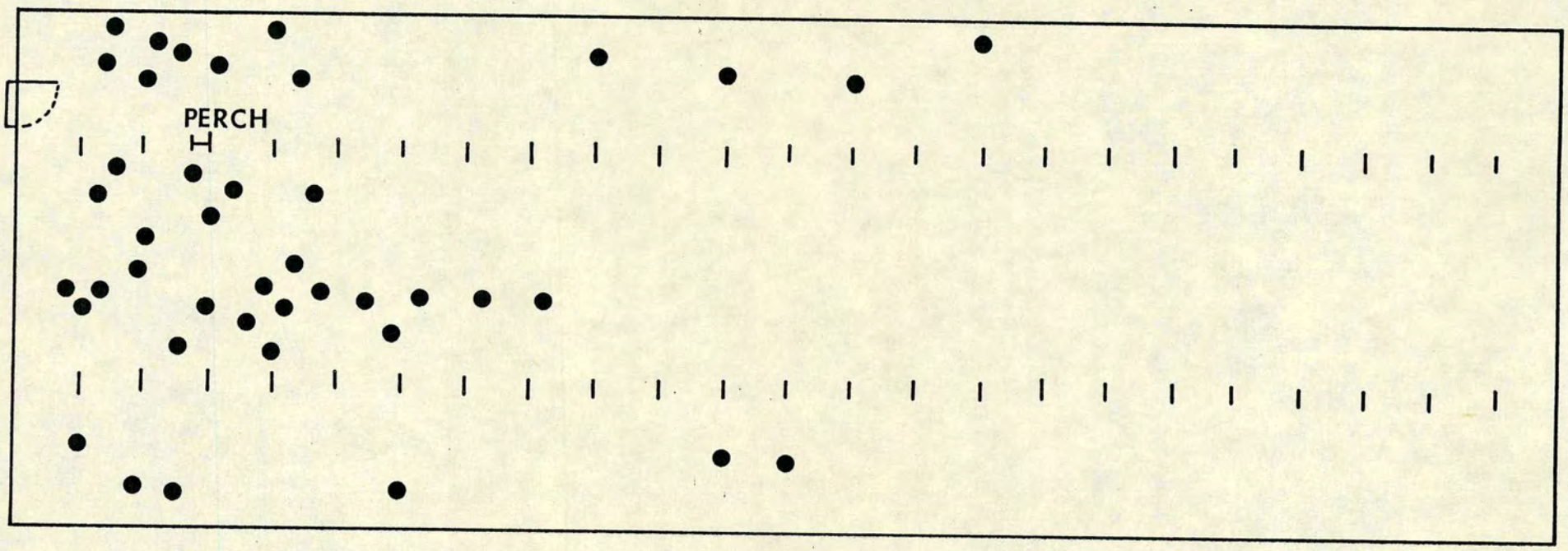
Information of the shedding of salmonellas by the birds cannot readily be obtained within commercial conditions and so our knowledge of the excretion of salmonellas is mainly based on data from laboratory trials carried out in connection with other facets of the "salmonella problem". These investigations have shown that when an inoculum of more than 5.0 salmonella/bird is administered orally to birds a high level of infection is established in the flock which reduces exponentially over their lifetime. On the other hand if birds become infected after contact with carrier birds the proportion of birds shedding salmonellas rises in the first 10 to 20 days after exposure and this level of infection is then maintained for at least a further 20 to 30 days (Smith & Tucker, 1980).

The pattern of shedding in all cases is similar whether the birds are first infected at 4d or 46d of age (Smith & Tucker, 1980a and b), is independent of breed (Milner & Shaffer, 1952; Smith & Tucker, 1975; McBride et al., 1978) formulation of the feed (Smith & Tucker, 1975) and the nature of the floor on which the birds are reared (Henderson et al., 1960; Olesuik et al., 1973; Smith & Tucker, 1975) and season (McBride et al., 1978). Stress especially by deprivation of water before exposure to infection or the transport of birds does increase the percentage of the birds excreting salmonellas (Sadler et al., 1969; Brownwell et al., 1969; Bhatia & McNabb, 1980).

The numbers of salmonellas recovered from the faeces of infected birds may vary from as many as 7.00/g (Wienach et al., 1979) to so few that they can only be detected by an enrichment method (Smith &

FIG.1.3

Positions of birds with spray marks, observed 36 hours after start of spraying.
(Turner, unpublished data 1981)



Tucker, 1975 and 1978). It has also been shown that the number of salmonellas excreted by each bird is not related to the number of infected birds in the group (Weinach et al., 1979) but generally the number of salmonellas in the faeces decreases with time after infection (Gibbons and Moore, 1946; Smith and Tucker, 1980a). However, as the weight of faeces produced by the birds is proportional to the body weight the total number of salmonellas shed onto each unit area of litter may well remain relatively constant.

In a broiler house those birds shedding salmonellas will contaminate their immediate environment and therefore the pattern of movement of these birds within the house is important. McBride et al. (1963) suggested that birds will maintain an area around themselves which has to be interpreted as a disease zone (Dimond, 1970). In small flocks in large pens it has been shown that movement of individual birds ranged from random movements throughout the pen to a preference to a particular part of the pen (Hughes et al., 1974) but under modern commercial conditions with a bird density of 0.47 birds/m^2, Turner (personal communication) showed that in a trial including the automatic weighing of birds all the birds which had visited a marked perch over the period of 3 h were distributed at random throughout the house within 36 h (Figure 1.3). Therefore each bird excreting salmonellas has the potential to contaminate any part of the broiler house, hence it may be concluded that "the carrier bird is probably the major factor in perpetuating infection" (Duff et al., 1973).

(b) Feedstuff

In a vertically integrated enterprise the feed ingredients are the major items which are continually being introduced into the unit and then disseminated throughout the system (Figure 1.4). Hence a great deal of work has been carried out on the incidence of salmonellas in feedstuffs, this has shown that many of the individual ingredients may be contaminated either at source or at the feed mills (Kauffman & Feeley, 1968; Pennington et al., 1968; Woodburn & Sladelman, 1968; Morris et al., 1969; Kumar et al., 1971; Bains & MacKenzie, 1974; Vaughan et al., 1974; Dougherty, 1976; Hacking et al., 1978; Bhatia & McNabb, 1980; McGarr et al., 1980; Higgins et al., 1982; Barbour & Nabbut, 1982). The fact that salmonellas are rarely isolated from compounded feed may be due to the difficulties encountered when sampling such a large bulk of material and the limits of the isolation techniques. It has been shown that as few as one salmonella per 2g of feed is sufficient to establish infection and that the contamination need only be presented once to cause infection in the flock (Gordon & Tucker, 1965).

While the feedstuffs are an obvious mode of introducing a serotype to a flock it is not always possible to confirm that they were the vehicle of the infection into the enterprise. For instance Timoney et al. (1970) showed that of 11 outbreaks of infection by Salmonella tenessee which they investigated in only two instances could the feedstuffs be implicated with any certainty, likewise Kumar et al. (1971) could involve feedstuffs in only 6 of the 9 infections which they studied and then definitely confirm just two of these sources.

It would therefore seem possible that the over-emphasis in the past on feedstuffs has led to a great number of surveys and has influenced a great deal of the conclusions drawn which has overshadowed the possibility that other sources of salmonellas could be of equal importance. Undisputedly there has been cases where feedstuffs have been the primary cause of infection but there are also cases in which these conclusions have been drawn using retrospective samples (Pennington et al., 1968).

Animal protein is the ingredient which is frequently found to be contaminated with salmonellas. Introducing the Protein Processing Order 1981 (Statutory Instrument 1981a) and the associated Protein (Import) Order 1981 (Statutory Instrument 1981b) feedstuffs should no longer give concern as sources of salmonella in the industry. These Regulations require that all animal protein to be fed to any class of livestock must be salmonella-free. As the animal protein for poultry used is often the waste products of the poultry processor such as feather, guts, feet, blood etc. the prescribed treatment, should prevent the recycling of salmonellas within individual enterprises.

(c) Water

Chickens drink a considerable amount of water, for example at 7d old, 100 chicks will drink 18 to 27 l/d while at 42d the volume is estimated at 113 to 117 l/d/100 birds (Anon, 1977a). Salmonellas have been recovered from the drinking water (Gauger & Greave, 1946; Snoeyenbos et al., 1967; Olesiuk et al., 1969; Morris et al.,

1969; Smyser et al., 1966; Woodburn & Stadelman, 1968; Dougherty, 1976; Hacking et al., 1978; Patterson & Gibbs, 1977; McGarr et al., 1980; Morgan-Jones, 1980; 1982; Barbour & Narbut, 1982; Higgins et al., 1982). In contrast Kumar et al. (1971) and Duncan & Adams (1972a) failed to isolate salmonellas from these sites.

Gordon & Tucker (1965) suggest that salmonellas in the drinking water could be controlled using a soluble chemotherapeutic agent. These workers together with Tucker et al. (1975) and Morgan-Jones (1980; 1982) have stressed the importance of water as a source of the primary and subsequent re-infection of chickens. When the water is contaminated it has been shown that the number of birds carrying salmonellas doubled and the period of time when they excreted salmonellas increased (Gordon & Tucker, 1965). Also at the Houghton Research Station Tucker et al. (1975) showed that when birds were placed on litter contaminated with Salmonella virchow this serotype was recovered from the water troughs within 48h. Ineffective disinfection of the water trough has been shown to result in a carry-over of S. worthington from the previous flock to a new flock of turkey poults (Morgan-Jones, 1982). The daily cleaning of waterers was found by Guager & Greaves (1946) to be ineffective so long as infected birds remained. It would therefore appear that water could be an important method of transmitting salmonella infection which is not yet fully recognised.

(d) Litter

Salmonellas have been isolated from straw (Bhatia & McNabb,

1980) and sawdust (Snoeyenbos et al., 1967; Hacking et al., 1978; Bhatia & McNabb, 1980) to be used as litter in poultry houses. Snoeyenbos et al. (1967) has even suggested that this material could serve frequently as a mode of carriage of salmonellas into the broiler house. Under the British system the fumigation of the house prior to occupation by birds makes it unlikely that new litter material is a common source of infection to day old chicks. However once infected chicks are placed on the litter salmonellas may be isolated from the litter within 24 hours (Bhatia & McNabb, 1980; Morgan-Jones, 1980; 1982).

Initially the numbers of salmonellas in the litter increase but as this material becomes more alkaline salmonellas are recovered less frequently (Botts et al., 1952; Rigby & Pettit, 1979; McGarr et al., 1980). In monitoring exercises Hacking et al. (1978), Weinach et al. (1978) and Morgan-Jones (1980; 1982) found that the recovery of salmonellas from litter was sporadic while Morris et al. (1969) failed to recover these bacteria from the litter samples which they examined.

Few workers have enumerated the numbers of salmonellas in litter. Botts et al. (1952) showed by a direct plating technique that the litter under chicks of 2d old had 4.48-4.58 salmonella per g and at 47d, 3.78-4.02 per g. Gordon & Tucker (1965) found the contamination rate in their litter to be in the same order of numbers (3.00-4.00 per g) but Rigby & Pettit (1979) showed a gradual decline 7.04 per g at 30d, 2.00 per g at 129d but at 163d salmonella could only just be detected.

On the advice of Snoeyenbos et al. (1970) many poultry managers sample litters from houses at 3-5w in order to detect the level of infection in the flock. These authors do suggest caution in the interpretation of the results but consider that the presence of salmonellas in litter indicates that subclinical disease is a problem in the flock.

The role of salmonellas in litter will be considered in further detail in other sections of this thesis.

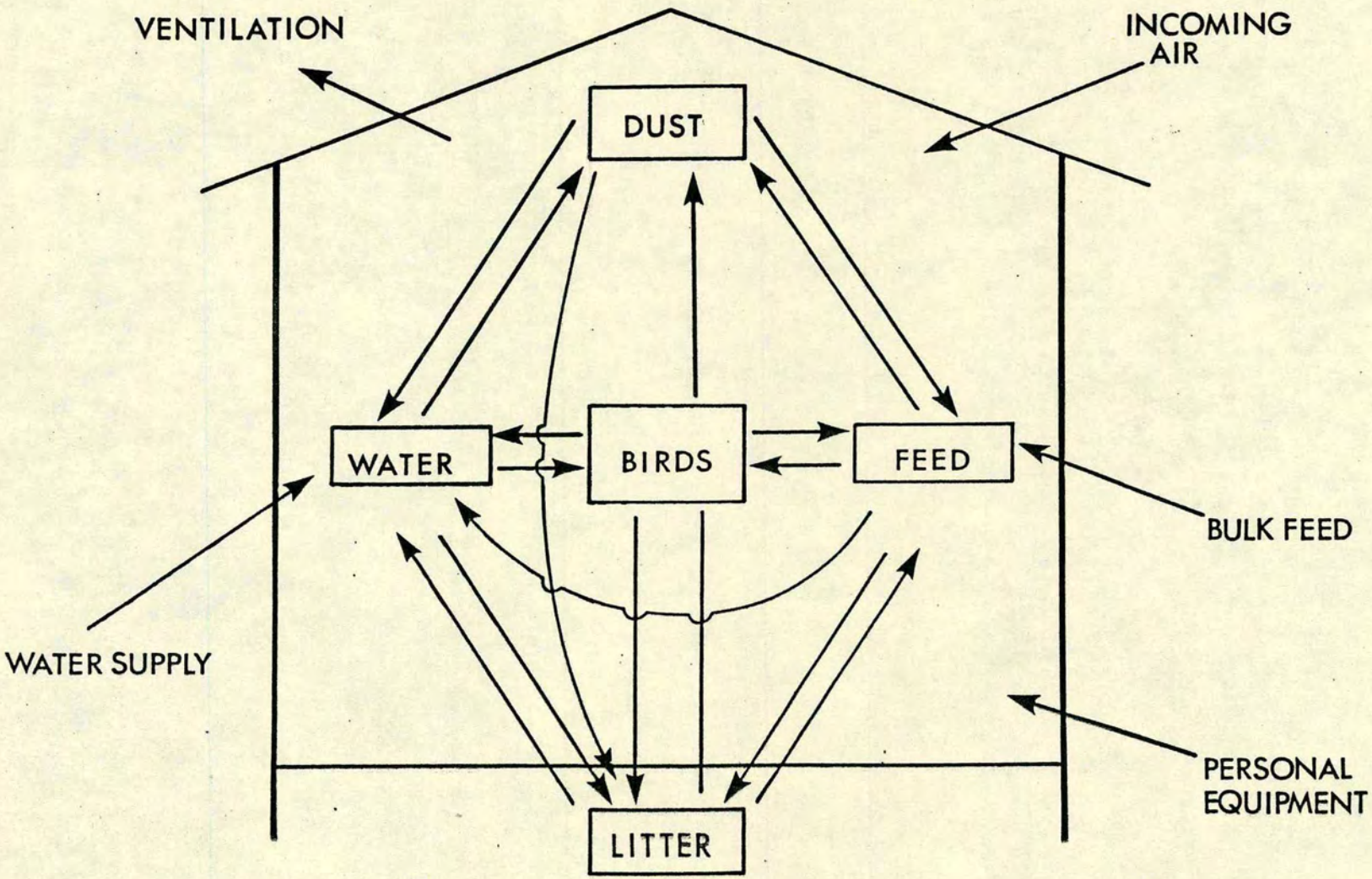
(e) Dust

The level of dust increases during the rearing period and gradually becomes deposited on all surfaces in the house. Initially the dust is derived from the feather spicules of the young chick and feed particles. Later particles of dried faeces and litter contributed a greater proportion of the dust although the feedstuffs remain the predominant constituent. Chemical analysis of the dust approximate to a typical analysis of poultry feed (Edwards, pers. com.).

The presence of salmonellas in dust has not been monitored as thoroughly as other materials from the broiler house and the results available present a confused picture. Smyser et al. (1966) recovered salmonellas from 12.8% of the samples they examined, Snoeyenbos et al. (1967) recovered 21.4%, Bhatia et al. (1979) 25.9%, Kumar et al. (1971) and Dougherty (1976) 40%, Higgins et al. 77.8%, Woodburn & Stadelman (1968) and Morgan-Jones (1980; 1982) 100% level.

FIG.1.4

CYCLE OF CONTAMINATION OF SALMONELLAS IN BROILER HOUSE.



(f) Vermin

While it is good practice to eliminate all vermin from poultry houses there is little evidence in the literature of such creatures carrying salmonellas. Mice, starlings and sparrows from the vicinity of houses known to be infected tested at this laboratory have never yielded these bacteria (Morgan-Jones unpublished data). The only report of mice carrying the infection is from Saudi Arabia when Salmonella concord, S. mbandaka, S. kentucky and S. montevideo were isolated from mice (Barbour & Nabbert, 1982).

Insects are another possible vector of disease in particular the Lesser Mealworm beetle (Alphitubus diaperinus) which was thought to carry Marek's disease before this disease was controlled by vaccination (Anon, 1982). These beetles are diurnal so they are rarely seen by farmstaff but can present a long term problem as they bore into the insulation. When grown in an artificially contaminated feedstuff they have been found to be infected with as many as 3-12 salmonellas per g sufficient to cause infection in a bird.

4. The Cycle of Contamination of Salmonellas in a Broiler House with Infected Birds

The cycle of contamination of salmonellas within a broiler house is a complex, dynamic process in which the relative importance of each component has not yet been fully evaluated. It is possible with a descriptive model (Fig. 1.4) to gain an impression of those areas which warrant further investigation.

The litter is primarily the bedding material for the birds but also provides minor but essential nutrients including some vitamins and trace elements (Schlamb & Winters, 1948; Mehner, 1952;

Gerriets et al., 1954; Kennard et al., 1954; Jacobs et al., 1958). At the early stages of rearing the litter can contribute 50-60% of the dust (Rollo et al., 1969). In addition the litter can be deposited in the feed and water by the beaks and feet of the birds. The faeces per se will also contribute to the dust but no quantitative data has been published on these aspects.

The level of dust in the house increases as the birds become older, the rate of deposition increasing from 0.008g/m²/d at 10d to 0.12g/m²/d at 42d (Morgan-Jones unpubl. data). Therefore dust will be deposited on the feed troughs so that 0.01% of the feedstuff consumed by the birds at 42d is deposited dust. However this dust has a chemical composition comparable to the feed so the performance of the birds is unaffected. If this dust is contaminated with salmonellas this could result in a previously salmonella-free feedstuff becoming contaminated within the house (Patterson & Gibbs, 1977).

In the case of water in the drinker even the addition of a minute quantity of dust will add sufficient nutrients to the water to enable salmonellas to multiply.

From this model it can be seen that the litter can act as an important reservoir for salmonellas in a broiler house for although mature litters are considered inhibitory to salmonellas it is possible to isolate these bacteria albeit in small numbers if the birds are infected. From the litter it can be seen that salmonellas can gain entry to the water, feedstuffs and dust and so will continue to cycle within the house.

D. The Aims of This Study

The search of the literature has shown that there is evidence that poultry litter becomes increasingly inhibitory to salmonellas during the rearing period of the broiler birds. This investigation aimed to confirm and elucidate the mechanism of this inhibitory action. A greater understanding of this system could identify these effects which may influence the epidemiology of infection or suggest a method of inhibition that might be used in a biological control of infection.

The deposition of litter from infected houses presents a serious public health problem therefore a simple method of rendering the material free of salmonellas could aid in reducing this environmental hazard.

No technique existed for enumerating salmonellas as a routine which is sufficiently economical in time and materials to be used in a routine laboratory so it was necessary to evolve such a technique before the above aims were persued.

CHAPTER II

General Materials and Methods

GENERAL MATERIALS AND METHODS

A. Cultures and Incubation Conditions for Maintaining Cultures

The cultures used in this study are detailed in Table 2.1. All strains were maintained on Nutrient Agar slopes in ½oz. Bijou bottles at 4°C and sub-cultured monthly. Stock cultures were also retained as freeze dried replicates. The cultures for the laboratory inoculation of litter were grown in 100ml of Nutrient Broth contained in 500ml conical flasks which were incubated at 37°C for 18h and then stored at 4°C in a domestic refrigerator until use on that day unless otherwise stated in the text.

Table 2.1 Strains of Salmonellas used in investigation and their source

Name	Serotype	Phage Type	Source	Reference/Source
Salmonella agona	4,12:gs:-	-	Poultry Litter	Morgan-Jones (1980)
Salmonella typhimurium	1,4,12:i: 1,2	104	Poultry carcase	Dr. C. Mills (D.B. Marshall, Newbridge)
Salmonella worthington	1,13,23:z:1, w	-	Poultry Litter	Morgan-Jones (1982)

B. The Source and Sampling of Poultry Litter

Except where specified in the text litter samples were collected from commercial broiler houses. The samples were taken by removing a small quantity of litter from the top 2cm of at least 50 sites evenly distributed within the house and bulking the sub-samples

in a sterile polythene bag, using polythene gloves to exclude contamination from the sample.

The age stated for each litter is that of the birds on the litter. Where a range of ages is given this indicates that the birds present were of more than one age.

The samples were transported to the laboratory as soon as possible and stored at 4°C until used.

All litters were of wood shavings or sawdust or a mixture of these wood products.

Data on the variability that may be anticipated between samples taken from individual houses will be discussed in Section 3.

C. Microbiological Analysis

(1) Bacteriological Media used for the culivation of Microbes

When possible Oxoid dehydrated media were utilised, preparation being by the method prescribed by the manufacturers. The formulation for the media prepared from ingredients at this laboratory and the details of supplies are detailed in Appendix I.

The media used, together with the abbreviation used in the text when appropriate were as follows:-

(a) Nonselective Media

(i) Brain Heart Infusion Agar (Brain Heart Infusion (CM225)) with 1.2% Agar No. 3 (L13) added) - BHI agar.

(ii) Buffered Peptone Water (CM509) - BP Water.

(iii) Diagnostic Sensitivity Test Agar (CM261) - DST agar.

(iv) Milk Agar (CM21).

(v) M_9 Broth (Gomaz et al., 1973).

(vi) Nutrient Agar (CM3) - N agar.

(vii) Nutrient Broth (CM1) - N broth.

(viii) Nutrient Broth No. 2 (CM67).

(ix) Plate Count Agar (CM325) - PC agar.

(x) Tryptone Soya Agar (CM131) - TS agar.

(xi) Tryptone Soya Broth (CM129) - TS broth.

(xii) VL Agar (Barnes & Impey, 1974).

(xiii) VL Broth (Barnes & Impey, 1974).

(xiv) VLhlf agar (Barnes & Impey, 1974).

(b) Selective Media

(i) Actinomycetes

(a) Czapek Dox Agar (modified) (CM97) with the addition of 2.5ml of 1.0% solution Novobiocin (BDH Biochemicals No. 44206) and 5ml of 1.0% Cycloheximide (Sigma Chemical Co., No. C-6255) - CX agar.

(b) ½ TS Agar

(ii) Bacteriodes group

(a) Ethyl Violet Azide Agar (Barnes & Goldberg, 1962) - EVA agar.

(b) VK Agar (Barnes et al 1972).

(iii) Cellulolytic Bacteria

(a) Cellulolytic Broth (Media File, Department of Microbiology, Univeristy of Edinburgh).

(b) Cellulose Broth (Mann, 1969).

(iv) Enterobacteriaceae

(a) Bismuth Sulphite Agar (CM201) - BS agar.

(b) Brilliant Green Agar (modified) (Cm329) - BG agar.

(c) Desoxycholate Agar (CM163) - DC agar.

(d) Lysine-iron-cysteine-neutral-red Broth (Hargrove et al., 1971) - LICNR broth.

(e) MacConkey Agar (CM7).

(f) Selenite Broth (CM395).

(g) Selenite-cysteine Broth (North & Bartram, 1975).

(h) SS Agar (CM533).

(i) Tetrathionate Broth (Heard et al., 1969).

(j) Tetrathionate Broth (Kauffman, 1930).

(k) Tetrathionate Broth (Rolfe, 1946).

(l) Violet Red Bile Agar (CM107) - VRB agar.

(m) XLD Agar (CM469).

(v) Methane and Methanol Bacteria

(a) NMR Broth (Whittenburg et al., 1970).

(vi) Streptococcus group

(a) Thallows Acetate Tetrazolium Agar (Barnes, 1956) - TAT agar.

(vii) Lactic Acid Agar

(a) MRS Broth (CM359).

(b) Rogosa Agar (Difco No. 0480-01-08).

(viii) Urea Decomposing Broth

(a) Urea decomposing Media (Schefferle, 1957).

(ix) Uric Acid Decomposing Bacteria

(a) Uric Acid Agar (Schefferle, 1957).

(ix) Yeasts and Moulds

(a) Malt Extract Agar (CM59).

(b) OAES Agar (Kauffman et al., 1963).

(c) Diluent

(a) 1/4 strength Ringer's solution (BR52) - Ringer's solution.

(b) Reinforced Clostridial Medium (CM149) - RCM broth.

(d) Confirmation of Salmonellas

(a) Kohn Medium No. 1 (CM179) - Kohn I agar.

(b) Lysine Medium (CM191).

2. Routine Analysis for viable Counts of Micro-organisms

(a) Plating Method for enumerating Bacteria

Viable counts for micro-organisms were obtained by spreading plate method. 0.1ml of the appropriate serial decimal dilution was spread over the surface of a dried agar plate with a sterile glass spreader. The prepared agar plates were dried either by storing at room temperature for 2-3d or in an incubator at 30°C for 18h. On a few occasions agar not containing inhibiting compounds were dried open at 60°C for a few minutes.

Incubation for the time and temperature detailed in the text later was carried out by at least one of the following:-

(i) Aerobic conditions

(ii) Anaerobic conditions using the Gas Pak System (BBL Microbiological System, Cockeysbille MD 21020, U.S.A.) observing the precautions detailed by Holdeman et al. (1977).

(iii) Micro-aerophilic conditions in an atmosphere of hydrogen containing 5-12% carbon dioxide and 5-15% oxygen (BBL Campyl Pak 11TM).

(b) Most-Probable Number Estimates of Bacteria

The most probable number method (MPN method) was used extensively in this work as preliminary experiments described later showed that the plate count method was likely to under estimate the actual number of these bacteria present. The main reason for the use of the MPN method was the difficulty of recovering salmonellas from amongst large numbers of other bacteria and to aid the recovery of bacteria which had been physiologically damaged.

To estimate the numbers of bacteria by the MPN method, 1ml quantities of each serial decimal dilution were inoculated into replicate solutions of the selected broth. After incubation the solutions were examined to determine the number of solutions at each dilution in which organisms had grown. Growth was determined either by the presence of turbidity, change of colour of an indicator chemical, or by streaking a loopful of the solution onto solid media. The number of bacteria in the original sample was determined by reference to the appropriate statistical table (Meynell & Meynell, 1973; Collins & Lyne, 1974).

The number of replicates at each dilution varied depending on the accuracy required for each determination, the larger the number of solutions used at each dilution the greater the degree of accuracy. In their comprehensive account of this method Meynell & Meynell (1973) quoted the standard error and confidence limits of the

estimate of count as determined by Cochran (1950). The figures which are shown in Table 2.2 show the order of standard error of the levels of replication selected for use in this investigation.

Table 2.2 Some Standard Errors of the MPN estimates at 3 levels of Replication (Cochran, 1950)

Number of tubes at each dilution	SE ($\log_{10}$) at the following dilution factors 2	4	5	10
3	0.174	0.246	0.265	0.335
5	0.135	0.191	0.206	0.259
10	0.095	0.135	0.145	0.183

This shows that while 5 solutions at each dilution is more accurate than 3 solutions the error involved is not great when compared to the total of errors in bacteriological analysis to warrant the additional media required. Therefore for routine purposes 3 solutions were inoculated at each dilution, at other times 1, 5 and 10 replicates being used when appropriate (MPN-1, MPN-3, MPN-5 or MPN 10 as appropriate).

(c) Turbidity method for estimating the growth of bacteria

The turbidity of the solution in a flaw-free test tube was measured by a Unigalvo Nephelometer (Corning Ltd., Holstead, Essex) with an orange filter.

3. Confirmation of Colonies characteristic of Salmonellas

Colonies with the appearance characteristic of salmonellas in mixed cultures on selective media were picked using a straight wire and inoculated with lysine broth and incubated at 37°C for 18h.

Those solutions showing growth but remaining purple were confirmed as containing salmonellas by inoculating a tube of Kohn I media. Solutions of lysine broth where the colour of the indicator changed from purple to yellow were further investigated by streaking a loopful of the solution onto a plate of McConkey agar. If the resultant growth after incubation at 37°C for 18h appeared to be a pure culture but not characteristic of salmonellas the plate was discarded as not being salmonellas. When a mixed culture was obtained, representative colonies were picked into lysine broth and retested as above.

The bacteria on the slopes of Kohn I media which were yellow after incubation at 37°C for 18h were confirmed as salmonellas by slide agglutination.

In experiments when salmonellas were added to sterile materials those colonies growing on the selective media were confirmed by the slide aggutination method only.

Slide agglutination was carried out by emulsifying a part of single colony in a drop of saline (0.85% sodium chloride in distilled water) on a clean glass microscope slide. One loopful of the appropriate serum (Wellcome Laboratories Ltd or Difco Co. Ltd.) was added and mixed into the emulsion. Agglutination clearly visible within one minute was recorded as a positive result.

D Chemical Analysis

(i) Formaldehyde

A preliminary trial showed that the Hartzsch reaction (Nash, 1953) was superior to Schiff reagent method (Schiff, 1966) and the 3-methylbenzothiazal-2-one hydrazone hydrochloride test (Knight &

Tennant, 1973) and so the first was adopted for the determination of formaldehyde concentration in both solutions and solid materials. Hartzsch reagent (Appendix 1b) was prepared 24h before use to enable stabilisation to take place and the solution discarded after 7d.

The concentration of formaldehyde was determined by adding 5ml of Hartzsch reagent to 5ml of the test solution or 5ml of a 10% (w/v) suspension of a solid material. A control solution of distilled water and Hartzsch reagent was prepared at the same time. After incubation at 37°C for 40 mins the amount of colour that developed was measured by a SP600 Spectrophotometer (Unicorn Instruments, Coatbridge) at 412μ wavelength set at 100% transmission with distilled water. The control solution must give a reading greater than 90% transmission.

A calibration curve was prepared for each batch of Hartzsch reagent by preparing a range of solutions from 1-8mg/l formaldehyde. The strength of the formaldehyde solution was determined accurately by titration of the iodine released from a 0.05M iodine solution with a standardised 0.1M solution of sodium thiosulphate in an acidic solution.

To ensure that the presence of wood products did not adversely affect this assay duplicate solutions of 10ml of distilled water containing known quantities of formaldehyde were prepared. One gram of wood shavings were added to one of the duplicates and both solutions stored overnight at room temperature. The level of formaldehyde was then determined at levels of formaldehyde from 0.4

to 8mg/l the results are shown in Table 2.3. At 0.1mg/ml of

Table 2.3 The effect of the Addition of Wood Shavings to the Assay of Formaldehyde by Hartzsch Reagent

Added formaldehyde (mg/l)	Spectrophotometer Reading (av. 3 recordings)	
	Control Solution	Extract of 10% wood shavings
0.1	14.2	8.0
0.4	17.8	16.1
0.8	28.5	31.5
8.0	66.5	66.1

formaldehyde the spectrophotometer reading of the solution containing wood shavings was lower than the corresponding control solution. It is possible that at these low levels some formaldehyde could have been bound onto wood products (Walker, 1964).

These results show that except for very low levels of formaldehyde the assay was accurate in the present of wood shavings.

2. Ammonia

(a) Demonstration of Presence of Ammonia

The presence of ammonia was determined by mixing one drop of the solution to be examined with one drop of Nessler reagent (Cowan & Steel, 1974) on a clean glass sheet. The intensity of the yellow-orange colour which is proportional to concentration of ammonia was noted and scored as follows:-

-	no colour change
+	pale yellow
++	orange
+++	orange-brown
++++	brown precipitate.

(b) Estimate of Ammonia concentration in Solutions of Bacterial Culture (Cunningham, 1947)

One ml of the culture was added to 10ml distilled water and one drop of methyl red indicator in alcoholic solution (0.08% w/v) added. The solution was titrated with N/14 sulphuric acid until the colour just began to change. The solution was then boiled and the titration completed when the solution was still hot.

1ml N/14 H_2SO_4 = 1mg ammonical nitrogen

(c) The Accurate Determination of Ammonia

The level of ammonia was determined using an ion-solution probe linked to a meter by the Nutrition Chemistry Department of the East of Scotland College of Agriculture.

3. pH value

The pH value of a solution or a 10^{-1} dilution as appropriate was determined by a Kent EJL 7015 pH Meter.

4. Moisture Content

A known weight of material (W_a) was dried at 100°c for 48h after which it was re-weighed (W_b). The moisture content was calculated as follows :-

$$\text{Moisture Content (\%)} = \frac{\text{Weight loss by heating}}{\text{Original weight}} \times 100$$

$$= \frac{W_a - W_b}{W_a} \times 100$$

E Fumigation by Formaldehyde

(a) Fumigation in the laboratory

The materials to be fumigated were laid loosely on a petri-dish or a sheet of paper on the shelf of a laboratory incubator of dimensions, 50cm x 50cm x 60cm. Formaldehyde was generated by adding 5ml of 37% formaldehyde solution onto 3.5g potassium permanganate in a high sided metal container. The door of the incubator was immediately closed and sealed with adhesive tape. After 18-48h the incubator was opened and allowed to ventilate naturally.

(b) Fumigation of Broiler Houses

The staff of the commercial units carried out the fumigation of the houses by their normal routine. No attempt was made to persuade these workers to modify their techniques. The methods used were as follows:-

(i) Heating paraformaldehyde

Paraformaldehyde pellets or granules (Jeyes Ltd.) were heated in a electrically heated pan designed for the purpose. 0.5kg of this solid was added to each pan which were placed not more than 30m apart. 100g of paraformaldehyde was used for each $30m^3$ of the house.

(ii) Aerosol of Formalin

Using an aerosol generator, 28ml of formalin was sprayed for each $3m^2$ of the building. The siting of the generators is critical to ensure that the whole building is misted with the liquid. It is also important that the generator produce a large size particle.

(iii) Monitoring the Effectiveness of Fumigation

A modification of the technique of Beery & Whitehouse (1965) used by the Microbiology Division of the Agricultural Development and Advisory Service was used to monitor the penetration of the formaldehyde. The test pieces were made by covering frames of 2cm mesh wire with films of domestic aluminium foil (Alcan Polyfoil Ltd., Amersham, Berks HP6 6JY) and sterilised by autoclaving at 121°C for 15min in a glass jar.

One drop of an 18h culture of Bacillus subtilis var globigii NC TC 10073 was placed in the centre of each test piece and dried on a warm heated plate. After drying each was transferred aseptically to a sterile glass jar with a plug of cotton wool at the base.

Before the house was fumigated the test pieces were removed aseptically from the vial and placed at sites throughout the houses. A control test piece was stored in the laboratory refrigerator.

On one occasion the culture was placed on the base of a plastic petri-dish instead of the test piece.

After fumigation the test pieces were placed individually in sterile vials and transported to the laboratory. At the laboratory each test piece was transferred to 9ml of Ringer's solution and

mixed with a Whirlmixer for 1 minute.

Dilutions were carried out to 10^{-4} and a plate count performed at each dilution. After incubation at 37°C for 2d a differential count of the known colonies characteristic of the indicator organisms was carried out.

F Determination of Antibiotic Activity by Filter Paper Disc Method

One ml of 10^{-1} dilution of an overnight culture of Salmonella typhimurium was added to 10ml of the specified agar and the "seeded" agar poured onto a 9cm petri-dish and allowed to solidify. The plates were then dried by leaving the plates open for 30min at 43°C. Filter paper discs (6mm) were dipped into the appropriate culture solution or aqueous extract and the excess liquid removed by touching the neck of the container. The disc was placed in a premarked position on the surface of the agar. The plates were incubated upright for 24h at 37°C after which susceptibility was judged by the criteria that a zone of inhibition of >15mm in diameter was recorded as positive inhibiton.

G Statistical and Graphical Illustrations

Statistical calculations were performed using the Minitab version 81.1 programmes (Pennsylvania State University, 1982) hence the statistical analyses are presented in the terms as defined in the Minitab Student Handbook (Ryan et al., 1976).

Graphical illustrations were executed using the Easygraph package (ERCC, 1983) and the Curvefit package (ERCC, 1983) envoked to calculate the possibility of a mathematically significant curve to the data.

All computer programmes were mounted on the 2900 ICL Computer of the Edinburgh Regional Computing Centre.

The statistical tables of White et al. (1979) were consulted to interpret the statistical analyses.

H Photography

A Leitz microscope with a phase contrast facility and a camera attachment containing Kodak S0115 film was used for black and white photographs. The films were processed at the Visual Aids Department of the East of Scotland College of Agriculture. Colour photographs were taken with Kodak Ektochrome ED 132-20 film using an Ilford Sportsman Camera or a Pentax ME-super camera. Photographs from other sources are acknowledged in the text.

I Expression of Results

All bacterial numbers were converted to their respective logarithms to the base 10 corrected to the second place of decimals. All computations were based on the $\log_{10}$ transformation.

Physical parameters were expressed untransformed unless stated otherwise in the text.

All results are expressed in terms of the wet weight basis.

CHAPTER III

The Enumeration of Salmonellas in Poultry Litter

ENUMERATION OF SALMONELLAS IN POULTRY LITTER

A Review of Literature

(1) Isolation Media

The presence of salmonellas in any material always merits concern. The methods for the isolation of salmonellas are complex (Harvey & Price, 1974) and so of necessity most workers design their procedures to establish the presence or absence of these bacteria in their sample without considering the number of salmonellas present. Moreover the research in the methodology of the isolation of salmonellas has concentrated on improving the efficiency of isolating these bacteria from a wide range of materials. As a result a large number of media have been advocated (Galton et al., 1968; Litchfield, 1973; Edel, 1974) but as none are completely reliable most investigators use at least two media in their testing schemes. However enrichment broths based on sodium biselenite and tetrathionate solution have been the most commonly used for detecting salmonellas in poultry-related materials. Selenite broth was used by Tucker et al. (1975), Smith & Tucker (1980); Morgan-Jones (1980 & 1982) and Barbour & Nabbut (1982) while Olesuike et al. (1969 & 1971); Snoeyenbos (1969); Kumar et al. (1971) increased the selectivity by adding Sulfa-pyridine to the medium, but brilliant green was used in addition by Snoeyenbos et al. (1969); Sato et al. (1971) and Bhatia & McNabb (1980) and cysteine by Woodburn & Stadelman (1968). Tetrathionate broths have become increasingly popular, this broth being used without additives by Vaughn et al. (1974) and Morgan-Jones (1980 & 1982) with the addition of brilliant green

by Fannelli et al. (1970); Duff et al. (1973); Bhadia & McNabb (1980); Higgs (1980) and Long et al. (1980) with novobiocin by Simmons & Byrnes (1972); Hacking et al. (1978) and McGurr et al. (1982); sodium nalidixate by Williams (1980). In conjunction with these enrichment broths a variety of plating media have been reported. Brilliant green agar was selected by Olesuike et al. (1969 & 1971); Snoeyenbos et al. (1969); Fannelli et al. (1970); Kumar et al. (1971); Sato et al. (1971); Vaughn et al. (1974); Bhatia & McNabb (1979) and Morgan-Jones (1980 & 1982); Sulfapyridine at 1% level being added by Simmons & Byrnes (1972); Hacking et al. (1978); Rigby & Pettit (1979); Bhatia & McNabb (1980); Long (1980); McGurr et al. (1982) and Higgins et al. (1982) and sodium naladixate by Smith & Tucker (1975 & 1980). Desoxycholate agar was the medium chosen by Vaughn et al. (1974); Tucker (1975a & b); Smith & Tucker (1980) and Morgan-Jones (1980 & 1982). This medium was enriched by the additon of sucrose (1%) by (Tucker,1975) who earlier also added 1% of salicin Tucker (1968; Duff et al., 1973 and Maestrone 1974) were the only workers to select MacConkey agar, the latter investigators increasing the selectivity by the addition of sodium naladixate. SS agar was used by Botts et al. (1952) and Vaughn (1974), Bhatia & MacNabb (1980) and Long et al. (1980).

Prior to 1968 the majority of workers incubated their enrichment broths at 37°C but since Harvey & Price (1968) advocated the use of an elevated temperature of 43°C most experimenters have adopted this temperature for all routine work.

The possibility that enrichment broths normally used for the

selection of salmonellas could be toxic to physiologically damaged cells was first shown by Thompson(1953). Since this date many workers have added a pre-enrichment stage to their method of isolation. Pre-enrichment is carried out by adding the material to a nutritious broth incubated at 6-24h at 37°C after which an aliquot of the solution is transferred to an enrichment broth (Harvey & Price, 1974; Edel & Kampelmacher, 1969 & 1970). The broth advocated for pre-enrichment for material from poultry enterprises have included 0.1% peptone water (Tucker et al., 1975a & b; Bhatia & McNabb, 1980), peptone water (Rigby & Pettit, 1979), peptone water with teepol (Higgins, 1972) buffered peptone water (Tucker et al., 1975a & b; Dougherty, 1976; Morgan-Jones, 1980 & 1982), lactose broth (Woodburn & Stadelman, 1968; Hacking et al., 1978) with trypticase broth and nutrient broth being used in parallel by Long et al. (1980). These workers all incubated their solutions at 37°C but Simmons & Byrnes (1972) incubated at 43°C a solution of peptone water containing teepol and brilliant green however as they were using 200 l quantities it is possible that this temperature was not achieved throughout the solutions in practice.

(2) The Enumeration of Salmonellas in Poultry Litter

As already stated very few investigators have attempted to enumerate salmonellas in their materials as in general the only available technique is MPN method based on media normally used for the detection of salmonellas. The only way of confirming the presence of salmonellas being to streak a loopful of each broth over solid

selective media, a method both cumbersome and expensive (Litchfield, 1970). However the earliest group to enumerate salmonellas in litter by a plate method were Botts et al. (1952) who plated the appropriate dilutions of litter onto SS agar but they do not give any data to confirm the proportion of salmonellas which they recovered. It is noteworthy that they used very dry agar plates which may have increased the toxicity of the media to the indigenous flora. When Duncan & Adams (1972) used brilliant green agar and a plating technique to enumerate salmonellas which had been inoculated into untreated and sterilised litter the plates from the untreated samples were overgrown with the indigenous flora of the litter. Tucker (1975a & b) found that the use of a modified Miles & Misra technique with desoxycholate agar was satisfactory in his hands. However in the same laboratory Smith & Tucker (1978) chose brilliant green agar with the addition of sodium nalidixate to ascertain the number of nalidixic acid resistant strains of Salmonella typhimurium. They also found that this medium was not specific to resistant strains alone as they were able to isolate 13 "wild" type serotypes with this medium (Smith & Tucker, 1980). Throughout their investigations these workers always use the term "estimate" rather than "enumerate". On the other hand Rigby & Pettit (1979) claimed to quantify salmonellas by adding 1ml of each decimal solution into one tube of tetrathionate-brilliant-green broth confirming the presence of salmonellas by plating a loopful of each solution on brilliant green agar and then express these results as $\log_{10}$ of salmonellas per gram. One of the few groups to

publish the detailed evaluation of this enumeration method are Woodburn & Steadelman (1968) who used a MPN-3 method to recover salmonellas added to poultry litter. After pre-enriching a sub-sample in lactose broth for 2h at 37°C they then transferred an aliquot to selenite-F broth. After incubation the presence of salmonellas was determined by streaking a loopful of the enrichment broths on MacConkey agar. When they assessed the accuracy they found that the number of salmonellas recovered by this technique was much lower than the original inoculum and so they declined to publish the data in their paper. The most complete evaluation of quantitative recovery of salmonellas is published by Li Cardi & Patten (1970) who studied thepersistence of salmonellas in skim milk. They pre-enriched three samples in water containing brilliant green. After incubation of 18-24h at 37°C, 1ml of this solution was added to tetrathionate broth containing brilliant green and sodium sulfathiazole and after a further 24h at 37°C a loopful of this soution was streaked on brilliant green agar also containing sodium sulfathiazole. They published the results of the MPN method together with plate count of the inoculum of S. typhimurium, and S. thompson after culturing at 6h, 24h and 48h in the skim milk powder. Using this published data the two methods can be shown to be in good agreement ($P = 0.005$). However, it should be remembered that the recovery of salmonellas from skim milk present few problems compared to those encountered in poultry litter as in skim milk the indigenous microflora is a relatively low number of thermoduric bacteria which probably do not grow rapidly under these conditions.

B Choice of Strain of Salmonellas as Experimental Inoculum for Poultry Litter

At the commencement of this investigation the use of a strain of salmonella with multiple resistance to antibiotics was considered as these bacteria can be recovered relatively easily by adding the appropriate chemical agents to the solid medium. However, the use of such a strain was discarded as the aim of this project was to determine the mode of inhibition in poultry litter where there was a possibility that antibacterial substances may be involved. It was possible that by chance the mutant selected for this investigation could be insensitive to such inhibitory substances. Other considerations of less weight were the stability of the resistance factor both long term in vitro and in short term experiments and whether such artificially induced strains behave physiologically in litter in the same manner as "wild" strains. There is ample evidence from animal experiments that following withdrawal of antibiotics that the strains of bacteria demonstrating acquired resistance rapidly disappear.

The foregoing account of the methods for quantifying salmonellas has shown that there is no established technique for the enumeration of salmonellas. Hence the decision to use a "wild" strain necessitated the development of a method of enumeration suitable for routine analysis which was sufficiently economical, both in time and materials, to enable a large number of samples to be examined in a working day.

C Experiment Work

1. The Selection of Medium to Enumerate Salmonellas in Poultry Litter

The prime requirement of a method for the enumeration of

salmonellas in litter must be a technique which yields maximum number of recoverable organisms consistently and is easily undertaken as a routine laboratory technique.

(a) Direct plating of litter artificially inoculated with Salmonella typhimurium on Selective Agars (Experiment 3.1)

Experimental Details

Eight litters (L1-8) from birds 25 to 56d old were sampled and within 18h of collection 10g of each weighed into a 250ml capacity glassjar. One ml of a range of decimal dilutions of S. typhimurium added dropwise to aliquots of the litter. After shaking by hand for 3 min the jars were incubated at 26°C for 24h when decimal dilutions of the inoculated litter from 10^{-2} to 10^{-6} prepared and 0.1ml of each dilution spread on previously poured and dried plates of the following media: BG agar, DC agar, SS agar, bismuth sulphite agar and XLD agar.

The inoculated plates were incubated at 37°C for 24h or 48h dependent on the medium.

Results and Discussion

Examination of the plates showed all to yield a profuse growth of colonies of contaminating bacteria so that colonies with the characteristics of salmonellas would not be readily detected. Even at high dilutions of litter large numbers of bacteria derived from the litter grew on the inhibitory media. These results indicated that conventional media are unlikely to be sufficiently selective to allow the quantitative recovery of salmonellas from poultry litter.

(b) Preliminary Screening of a Variety of Combinations of Selective Broths and Solid Media (Experiment 3.2)

LICNR broth has the ability to indicate the presence of salmonella by a colour change this media was chosen in comparison with selenite broth (Oxoid Ltd.) and the tetrathionate broth of Rolfe (1946) which the author had used in previous investigations (Morgan-Jones, 1980 & 1982), together with the selenite broth with added cysteine (North & Barton, 1975) and two formulations of tetrathionate broth viz. - Kauffmann (1970) and Heard et al. (1969). Five plating media viz. BG Agar, DC agar, bismuth sulphite agar, SS agar and XLD agar were used as the solid phase for the determination of the presence of salmonellas in the enrichment broth.

Experimental Details

The evaluation of the plating and enrichment methods were performed in parallel with Experiment 3.1. Therefore the decimal dilutions of litters (L 1-8) already described, were used to inoculate the enrichment solutions. One ml quantities of each dilution were added to five replicates of 10ml quantities of each solution under test in $^{5}/_{8}$" test tubes for each level of dilution. After incubation for 48h at 37°C a loopful of each solution was streaked on previously dried plates of each medium. After incubation for 24h or 48h at 37°C as appropriate for the medium, the plates were examined by the presence of colonies with the appearance characteristic of salmonellas. Representative colonies confirmed by the technique described in Chapter II.

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Results and Discussion

No permutation of enrichment broth and plating media was outstandingly successful (Table 3.1).

Table 3.1

Comparison of a Range of Enrichment Broth and Solid Media for the isolation of Salmonellas from Litter (L 1-8)

Enrichment Broth	As formulated by:-	No. of positive samples with isolation of salmonellas					
		Brilliant Green Agar	Desoxy-cholate Agar	SS Agar	Bismuth Sulphite Agar	XLD Agar	% positive
Tetrathio-nate Broth	Kauffman (1930)	6	2	1	4	3	33.33
Tetrothio-nate Broth	Rolfe (1946)	7	6	2	5	4	50.00
Tetrathio-nate Broth	Heard et al. (1969)	4	3	2	0	0	18.75
Selenite Broth	Oxoid	4	0	3	0	0	14.58
Selenite-cysteine Broth	North & Bartram (1975)	3	0	2	0	0	10.42
LICNR Broth	Hargrove et al (1971)	6	5	5	5	1	45.84
% positive		62.5	33.33	31.25	29.17	16.67	

However, when the number of occasions on which S. typhimurium were totalled, it was found that the tetrathionate broth of Rolfe (1946) and LICNR broth appeared superior to the other three enrichment broths. BG agar was the most successful solid medium.

(c) Comparison of Tetrathionate Broth (Rolfe, 1946) and LICNR Broth for the Isolation of Salmonella typhimurium from Litter (Experiment 3.3)

In the initial trial the numbers of salmonellas inoculated into the litter was not known. In this experiment the ability of the two most promising media to recover a known number of salmonellas added to a litter was assessed.

Experimental Details

Samples of five litter (L 9-13) were obtained from houses containing birds of 42 to 56d. Ten grams of each litter was inoculated with 1ml of an overnight culture of S. typhimurium shaken by hand for 1 min when 90ml of Ringer's solution was immediately added. Decimal dilutions of up to 10^{-10} were prepared, 1ml of each dilution was then inoculated into 5 replicate solutions of each broth. The solutions were incubated at 37°C for 48h when the presence of S. typhimurium was determined by streaking a loopful of each solution in BG agar. After the plates had been incubated at 37°C for 24h they were examined for characteristic colonies which were confirmed as S. typhimurium. The number of salmonellas in the litter was determined from probability tables.

The number of salmonellas in the original culture were enumerated by plating directly on BG agar.

Results and Discussion

The number of salmonellas as determined by BG agar was 6.9 bacteria/ml, by tetrathionate broth, 6.68 $\pm$ 0.22 bacteria/ml and

LICNR broth, 6.48 ± 0.29 bacteria/ml, results which are not significantly different (P = 0.05). (Table 3.2)

Table 3.2

Comparison of numbers of Salmonella typhimurium recovered by LICNR broth or tetrathionate broth (Rolfe, 1946) from Poultry Litter inoculated with a known number of bacteria

Litter	Estimate of Number of salmonella/g using LICNR Broth	Tetrathionate Broth
L5	6.26	6.95
L6	6.95	6.76
L7	6.54	6.54
L8	6.39	6.39
L9	6.26	6.74
Mean ± SD	6.48 ± 0.29	6.68 ± 0.22

Inoculum level = 6.9 bacteria/ml

D. The Confirmation of LICNR Broth as a suitable medium for the enumeration of Salmonella typhimurium in Poultry Litter

(a) The Inclusion of Novobiocin in LICNR broth (Experiment 3.4)

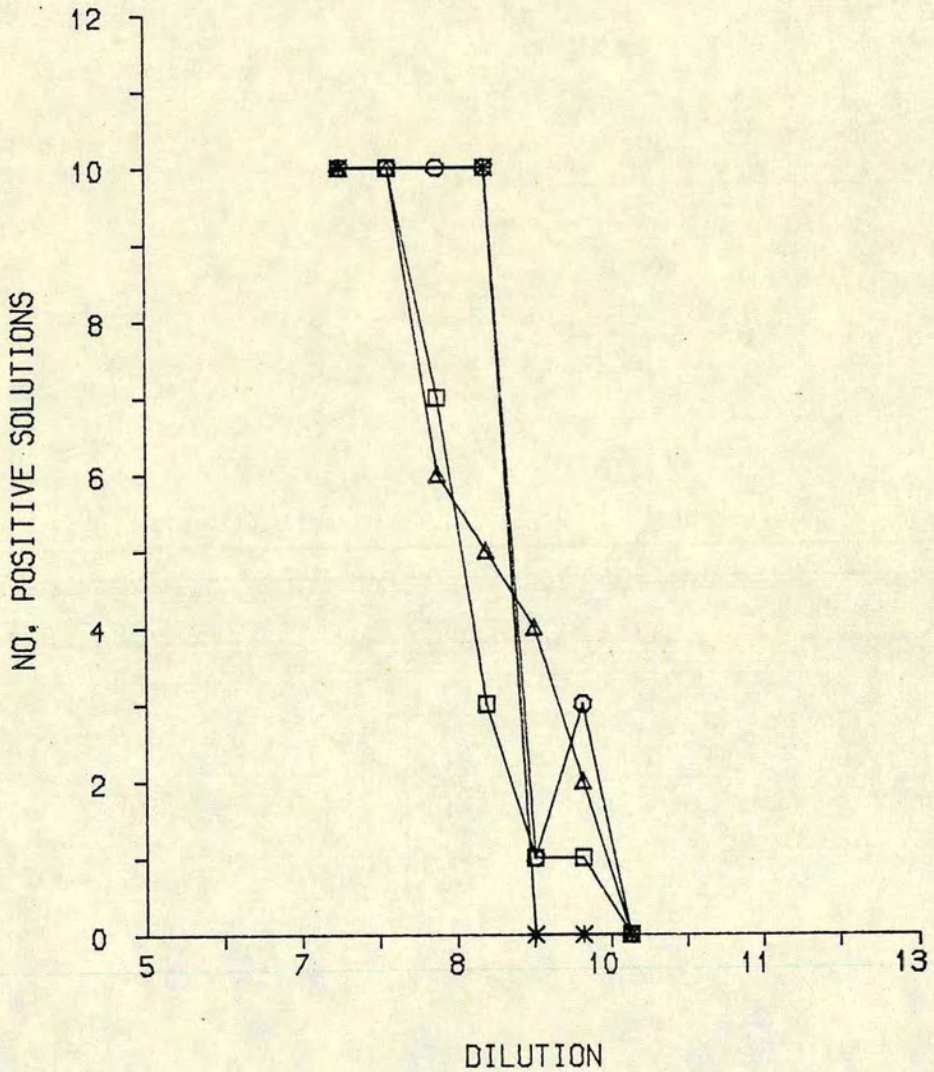
When Hobden et al. (1973) assessed LICNR broth for use with foods they reported that the addition of novobiocin increased the selectivity of the medium. This claim is now investigated in respect of poultry litter.

Experimental Details

The following solutions were used to enumerate the salmonellas in a culture by the MPN-10 method the solutions containing novobiocin (Sigma, Chemicals Ltd.) as follows:-

FIG 3.1. THE EFFECT OF THE ADDITION OF NOVOBIOCIN ON THE RECOVERY OF SALMONELLAS FROM LICNR BROTH.

(KEY. ○ NO NOVOBIOCIN, □ +NOVOBIOCIN,
✳ +NOVOBIOCIN+LITTER A, △ +NOVOBIOCIN+LITTER B)



(i) LICNR broth no additive

(ii) LICNR broth + 250mg novobiocin per 1000ml

(iii) LICNR broth + 250mg novobiocin per 1000ml + 10ml, 10% litter (PL 1) per 1000ml.

(iv) LICNR broth + 250mg novobiocin per 1000ml + 10ml, 10% litter extract ('L 2) per 1000ml.

Results and Discussion

As can be seen in Fig. 3.1 the advantage of adding novobiocin was not confirmed at this laboratory, an observation which has since been substantiated at Utrecht (Mossell, pers. comm).

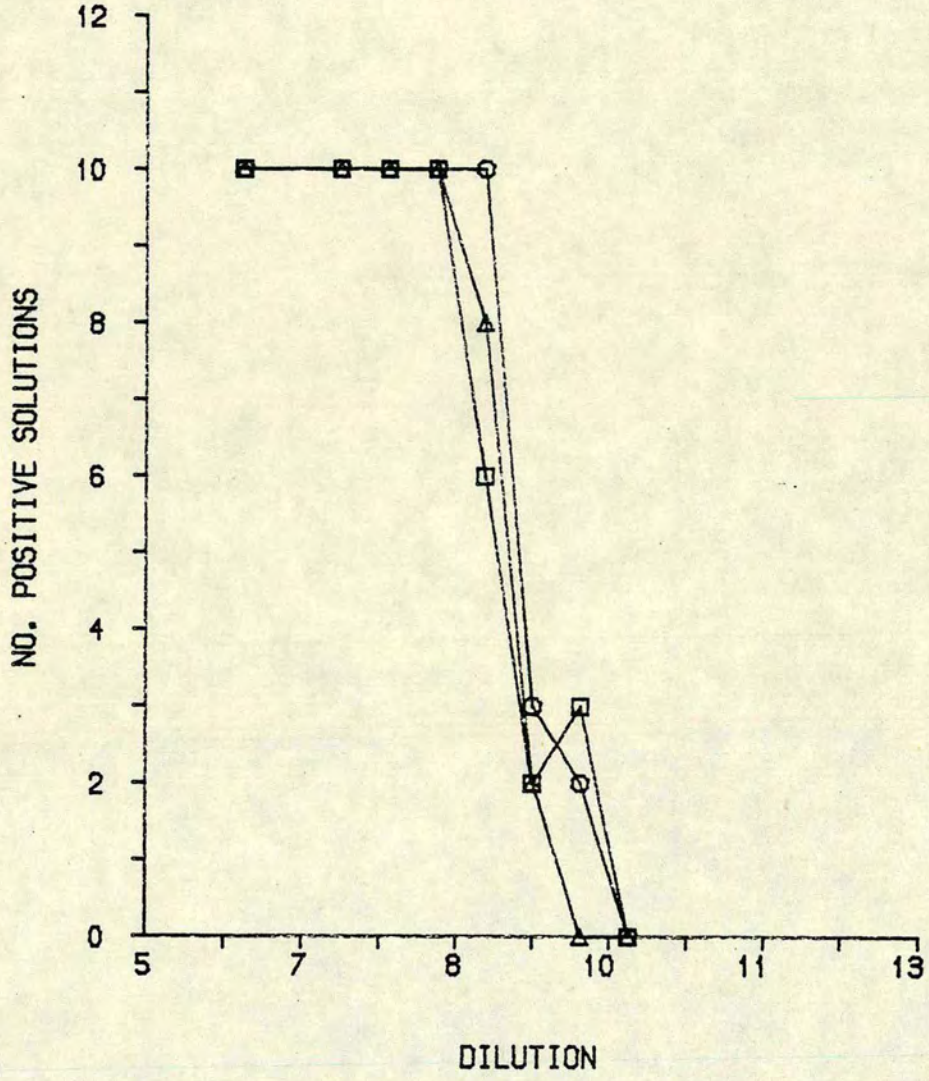
The inclusion of 5g/1000ml bile salt (Oxoid No. L55) 1, 5 or 10g/1000ml sodium desoxycholate (BDH Biochemicals Ltd., Poole, Dorset) 2g/1000ml sodium biselenite (Oxoid No. L121), 2 or 4ml/1000ml of the iodine solution of the tetrathionate broth of Rolfe (1946) were also found not to enhance the selective of this medium.

(b) Confirmation of the Efficiency of LICNR Broth to Enumerate a Variety of Serotypes of Salmonellas in the Presence of an Extract of Poultry Litter

LICNR broth was initially formulated for use with milk and dairy products, so the addition of poultry litter modifies the chemical composition of this medium and introduces a contaminating microflora very different from that of dairy products. Therefore it was necessary to confirm that the dose-response curve of the recovery of salmonellas from LICNR broth including poultry litter conformed to the Poisson Distribution to ensure that this media is suitable for use in a MPN

FIG 3.2. THE DOSE-RESPONSE CURVE OF THE ENUMERATION OF THREE SEROTYPES OF SALMONELLAS IN LICNR BROTH INCORPORATING POULTRY LITTER.

(KEY: □ S.AGONA, △ S.TYPHIMURIUM, ○ S.WORTHINGTON)



method (Meynell & Meynell, 1970). Therefore two experiments were carried out to confirm the suitability of LICNR broth for use with poultry. Firstly small numbers of salmonellas were added to LICNR broth containing an extract of poultry litter. Secondly the numbers of salmonellas and the concentration of poultry litter extract were both simultaneously decreased by increased dilution by using the conventional technique.

(i) The Dose-response Curve of the Enumeration of three serotypes of Salmonellas in LICNR Broth incorporating Poultry Litter (Experiment 3.5)

Experimental Details

The following litters were separately incorporated in LICNR broth at the rate of 1%(w/v)

L 10 - 14d

L 11 - 32d

L 12 - 46d

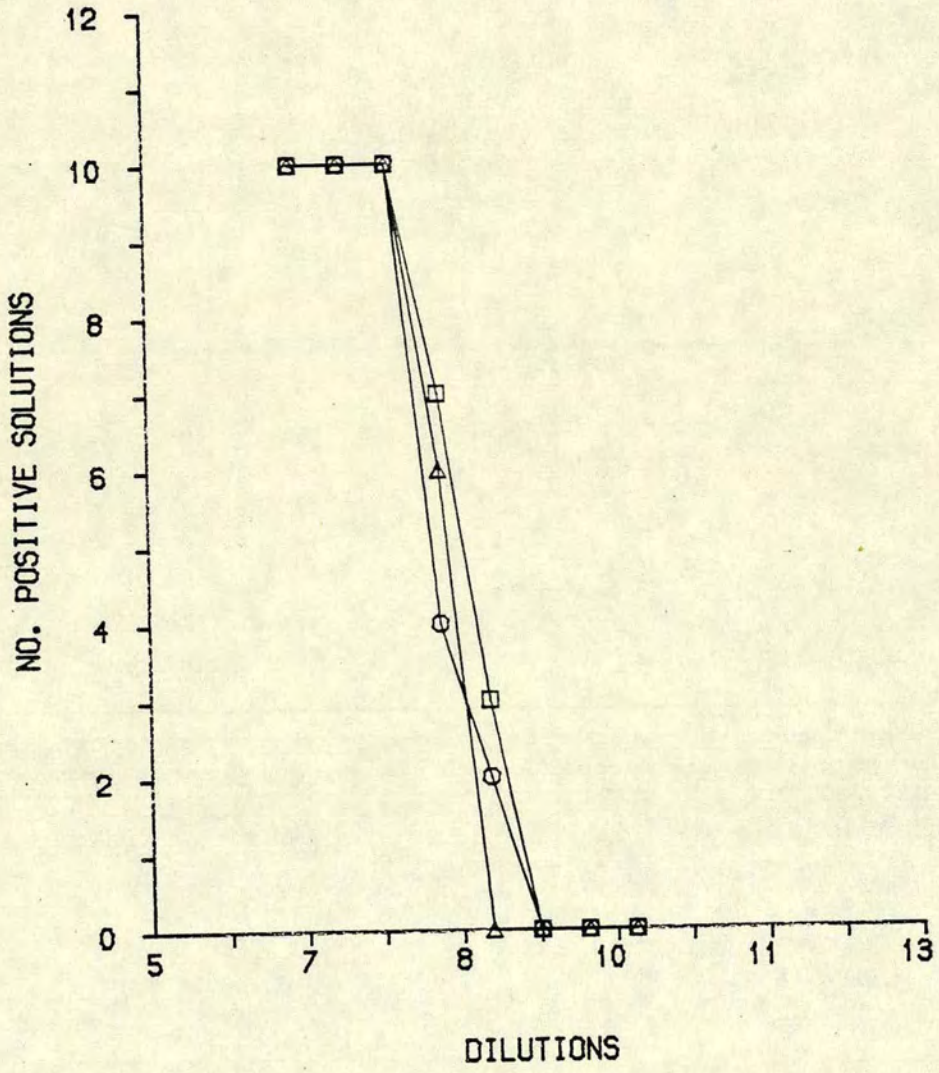
The following dilutions of Salmonella agona, S. typhimurium and S. worthington were prepared in Ringer's solution - 10^{-5}, 10^{-5}, $10^{-6.5}$, $10^{-7.5}$, 10^{8}, $10^{8.5}$, 10^{9}, $10^{9.5}$ and 10^{-10}.

One ml of each dilution was added to each of 10 tubes each containing 9ml of LICNR broth containing litter extracts as detailed above. After incubation at 37°C for 48h, one loopful of each broth was streaked on dried plates of BG agar.

The number of salmonellas in the original culture was determined by the plate count method on N agar.

FIG 3.3. THE DOSE-RESPONSE CURVE OF THE NUMBERS OF S. TYPHIMURIUM IN LICNR BROTH INCORPORATING LITTERS OF VARIOUS AGES.

(KEY ○ LITTER 6D, △ LITTER 14D □ LITTER 42D),



Results

When graphed (Fig. 3.2) the results show that the addition of the litter extract did not interfere with the recovery of salmonellas from LICNR broth. The number of salmonellas as determined by the plate count method gave a good agreement with MPN-10 method (Table A3 (ii) Appendix 2).

(ii) The Dose-response curve for the Enumeration of Salmonellas suspended in a Litter Extract (Experiment 3.6)

Experimental Details

Experiment 3.5 was repeated except that the inoculum used was 1ml of cultures in 9ml of a poultry litter extract (L 12) and the subsequent dilutions prepared in Ringer's solution.

Results

Fig. 3.3 shows that the dose response curves of this determination conforms to the Poisson distribution.

Discussion of Experiments 3.5 and 3.6

The inclusion of poultry litter does not affect the suitability of LICNR broth for the enumeration of salmonellas as judged by the agreement to the Poisson distribution. When the numbers of bacteria determined by the MPN-10 method and the respective plate counts are compared statistically they were found to be significantly different at the 5% level. The variation between the two estimates of the numbers in the original culture can be explained as the MPN-10 method gives a more accurate estimate than a single plate count.

In addition the broth method will allow the recovery of cells requiring resuscitation so the numbers of salmonellas determined by this technique will include some cells which would not be detected by the plating method.

(c) The Comparison of the Recovery of Salmonellas with LICNR Broth and a Method incorporating a Pre-enrichment Phase (Experiment 3.7)

The inhibitory effect of mature poultry litter to salmonellas might be anticipated to result in a proportion of the cell becoming physiologically damaged. Therefore the inclusion of a resuscitation phase of non-selective nutrient broth to allow these cells to repair may result in increased recovery of salmonellas when compared with incubation in enrichment broth alone. Although LICNR broth does not include ingredients which could be anticipated would be toxic to injured cells it was essential to assess whether cells of reduced viability are commonly present after exposure of salmonellas to litter. Should such an effect be proven it would clearly require note to be taken of it in the design of experiments involving the survival of bacteria in litter.

Experimental Details

The cells in 1ml of a overnight culture of Salmonella typhimurium and S. worthington were exposed to the effect of a 10% extract of 10 mature litters (L13-22)at room temperature for 3h. After this conditioning, decimal dilutions from 10^{-2} to 10^{-10} were prepared and the number of salmonellas was determined in LICNR broth using the MPN-5 method.

Simultaneously 1ml of each dilution was added to each of 5 aliquots of 10ml BP Water which was incubated at 37°C for 18h. One ml of each solution was then transferred to solutions of tetrathionate broth (Rolfe, 1946) which were incubated for a further 18h at 37°C after which a loopful was streaked on BG agar to determine the presence of salmonellas.

Results and Discussion

Statistically analysis of the results of the recovery of salmonellas from the raw data detailed in Table A3 (iii) in Appendix 2 summarised in Table 3.3 shows that there is no advantage to be gained by including a resuscitation phase.

Table 3.3

Summary of Statistical Analysis of Comparison of the Recovery of Salmonellas with LICNR broth and a Method incorporating a Pre-enrichment Phase (raw data in Table A3 (iv) in Appendix 2)

	F-value (16 degrees of freedom)	
	Bacterial count	LICNR Broth
LICNR broth	6.85*	-
Resuscitation method	6.72*	7.67*

Three hours may be a comparatively short period of contact with litter and could be insufficient time to damage the bacterial cells.

(d) The Feasibility of Storing LICNR broth as a concentrated stock solution (Experiment 3.8)

This project was instigated before a dehydrated formulation of LICNR

broth was marketed in Great Britain by Difco Ltd. As the medium required considerable time for preparation and quality control, the possibility of storing the medium in bulk as a tenfold strength stock solution was an attractive proposition. Such a solution would contain 30g mannitol and 50g glucose there was therefore the possibility of caramelisation of the sugars at autoclaving. The feasibility of such a modification of the medium preparation required investigation.

Experimental Details

Tenfold strength LICNR broth was prepared and divided into 10 equal portions and these autoclaved at 121°C for 15 mins. One bottle was selected at random and made up to 1 litre with distilled water. This broth was then used to enumerate the number of organisms in cultures of Salmonella agona, S. typhimurium and S. worthington. A count of the number of salmonellas in the cultures was undertaken on BG agar by the plate count method as a comparison.

Result

As can be seen in Table 3.4 there was no disadvantage in preparing the medium in this manner. In practice it was found that boiling the solution, cooling and storing at 4°C eliminated the need to autoclave the solution.

Table 3.4

The Recovery of Salmonellas determined by LICNR Broth stored as a concentrated solution and the corresponding Plate Counts

	Number of Salmonellas isolated by:-	
Serotype	MPN Method in LICNR Broth	Plate Count (av. of 2 plates)
Salmonella agona	8.96	7.59
S. typhimurium	8.22	8.81
S. worthington	8.44	8.74

(e) The Reliability of the Colour Change in LICNR broth as indicative of the presence of salmonellas

In the experiments already described the presence of salmonellas in the solution of the critical dilutions were confirmed by streaking a loopful of a solution on BG agar. The results of colour change and the culturing of 360 tubes were noted and assuming the growth of salmonellas on BG agar represents a positive isolation it can be seen that from Table 3.5 that only 3% of solutions would have been recorded as false positives and 8.3% as false negatives if the colour change was taken as the sole criteria.

Table 3.5

The Reliability of Colour Change of LICNR broth as indicative of the Presence of Salmonellas in Solutions (raw data in Table A3 (iv) in Appendix 2)

Accuracy by visual assessment*	Number of solutions examined	% of all solutions
Accurate	319	88.62
False positive	11	3.05
False negative	30	8.33

*Presence or absence on BG agar taken as proof of accuracy.

During all the experiments already described the inoculated solutions of LICNR broth have been examined visually daily. It had been noted that in the experiments in which salmonellas had been exposed to poultry litter there was a delay of 24-36h before the distinct colour change was observed. When pure cultures were assayed

this transformation had taken place by 24h of incubation. These observations suggest that either resuscitation may take place during the first 24-36h of incubation in this medium or that the inclusion of a small amount of poultry litter in LICNR broth inhibits the early multiplication of salmonellas.

In the foregoing experiments LICNR broth has been shown to yield results comparable with or superior to other media and to produce statistically accurate results under the conditions in this laboratory. but as argued by Smith (1952) the "seed" cultures have all been grown in laboratory media and may differ in behaviour to strains grown in vivo and excreted into the litter in the faeces and so could give an inaccurate result. Although a range of litters were used in these experiments litter is a very variable material and it is possible that the efficiency of isolation varies from litter to litter. However as no other medium proved to be more suitable than LICNR broth, this medium was selected as the standard broth in this investigation. The successful extension of the use of this medium to other products (Morgan-Jones, 1982) and the confirmation of the technique by Mossel and his co-workers at Utrecht (Mossel , pers. comm.) have served to increase confidence in the chosen methodology.

E Statement of the Chosen Method for the Enumeration of Salmonellas in Litter

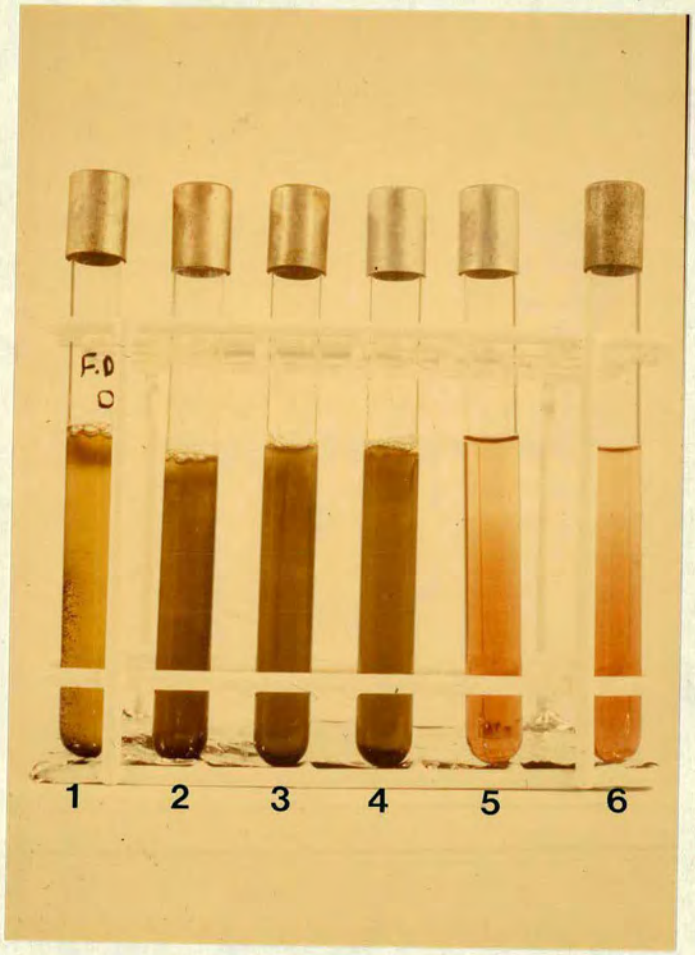
(a) Preparation of Medium

A concentrated solution of LICNR broth was prepared containing

Photograph 3.1

The Interpretation of the Colour Change of LICNR Broth after incubation with inoculum.

(Tubes 1-4: positive Tubes 5-6: negative)



L-lysine, 100g: tryptone, 50g: yeast extract, 30g: mannitol, 50g: glucose, 10g: salicin, 10g: ferric ammonium citrate (brown), 5g: sodium thiosulphate, 1g: L-cysteine, 1g: neutral red, 0.25g: distilled water, 1,000ml. After standing on a heated plate to dissolve the ingredients the solution was boiled and after cooling stored at 4°C. Two litre quantities of concentrated medium were prepared on each occasion.

Prior to use the concentrated solution is diluted to single strength with distilled water and the pH value adjusted to pH 6.2. Five ml quantities of this broth are then dispensed into ½in. tubes. Whenever possible the medium was used immediately, when this was not possible the solution was autoclaved at 121°C for 15mins and stored at 4°C.

(b) Examination of Samples by the MPN-3 Method

The appropriate decimal dilutions of the samples were prepared using Ringer's solution as diluent. Three solutions were inoculated for each dilution, the solutions incubated at 37°C for 48h.

After incubation the colour change of the solution was noted. The three replicates at the highest dilution showing colour changes were confirmed as positive or negative by streaking a loopful of each solution on one-third of a dried plate of BG agar. When possible the triplet chosen was one in which two of the three solutions showed a colour change indicative of salmonellas. On those occasions where the colour changes were ill-defined more than three decimal dilutions were verified.

The number of salmonellas in the sample was calculated from the appppropriate probability tables (Collins & Lyne, 1976).

(c) Expression of Results

Presumptive count of salmonellas per g or per ml as appropriate and always expressed as the $\log_{10}$ of the count obtained.

CHAPTER IV

THE SAMPLING METHOD AND THE ERRORS INHERENT IN SAMPLING POULTRY LITTER FROM BROILER HOUSES

THE SAMPLING METHOD AND THE ERRORS INHERENT IN SAMPLING POULTRY LITTER FROM BROILER HOUSES

A Review of Literature

A typical broiler house is approximately 40m long and 10-15m wide. Into this area opens a large number of doors used for catching birds for slaughter and for the removal of litter all of which are closed during the rearing period. In addition there is at least one door used regularly by the farm staff.

The temperature within the house ranges from slightly above ambient temperature at the walls and doors to 70-80°F at the centre. Therefore as the macro-environment varies greatly within the house it can be expected that there will be similar variations in the micro-environment within the litter. In such situations obtaining a representative sample presents a great problem. To date no standard method has been published for sampling poultry litter, but a large diversity of methods have been suggested. The majority of workers have reported taking small sub-samples at random throughout the house, the number of sub-samples varying from 1-2 (Sato et al., 1971), 2-4 (Tucker & Gordon, 1965; Tucker, 1967; Fanelli et al., 1970), 15 (Botts et al., 1952; Kumar et al., 1971; Long & Russell, 1980), 160-180 (Morgan-Jones, 1980) but many investigators do not specify the number of sub-samples collected. Again the method of sampling is not explained by many workers, however Fanelli et al. (1970) used forceps to collect chips, while Hacking et al. (1978), Bhadia et al. (1979) and Bhadia & McNabb (1980) removed small quantities of litter with tongue depressors, paper cups were used

by Williams (1980) and an aluminium scoop by Simmons & Byrnes (1972). The size of the sample examined at the laboratory varies from 0.25g (Fanelli et al., 1970) to 300g (Hacking et al., 1978).

The only workers to evaluate their sampling programme were Long & Russell (1980) who were concerned with the levels of oocysts in the litter. They found that the variability of counts was greatest at 2w when the amounts of droppings were very small, later the oocysts were found to be distributed at random in the house. While these results suggest that litter is uniform in nature the nature of oocysts is very different from that of microbes and therefore an evaluation of variability of litter in relationship to micro-organisms was essential before undertaking the sampling programme.

B Experimental Work

(i) The Effect of Weight of sub-sample on the Accuracy of Recovery of Salmonella typhimurium from Litter inoculated in the Laboratory (Experiment 4.1)

In the laboratory the levels of persistence of salmonellas can be examined either by inoculating a large quantity of litter and weighing sub-samples from this bulk at pre-arranged intervals for testing or by initially weighing out the sub-samples and inoculating these separately at zero time and then sacrificing a sub-sample at the given times. This experiment was carried out to contrast these two approaches and to determine whether the weight of the sub-sample is significant.

Experimental Design

Four litter samples were collected from poultry houses (L 27-30) and after thorough mixing five quantities of 1g, 10g and 100g of each weighed into appropriate sized glass containers. Each replicate was inoculated with the equivalent of 0.1ml per g of 10^{-4} dilution of S. typhimurium, after shaking by hand for 2 min the samples were incubated at 26°C for 2d. After incubation 1g quantity of the samples of 10g and 100g weight were weighed out and the number of salmonellas in these and the original 1g quantities determined by the stated MPN-3 method.

Results and Discussion

Analysis of the raw data (Table A4 (i) in Appendix 2) showed that the chosen litters were very different ($P = <0.001$) and therefore the litters were considered separately for statistical analysis. As can be seen in Table 4.1 there is a poor agreement between results whether judged by the mean values and standard deviations of the samples or by computing the F-values, therefore it was concluded that no sample size had an advantage when compared to the other sample sizes.

Table 4.1

Summary of Statistical Analysis of Number of Salmonellas per gram persisting in Litter as determined in sub-samples of three weights (raw data in Table A4 (i) in Appendix 2)

(a) Mean and Standard Deviations

Litter Number	Mean ± SD by weight of sample		
	1g	10g	100g
L27	0.39 ± 0.60	0.45 ± 0.43	1.47 ± 1.78
L28	3.13 ± 0.89	3.74 ± 0.91	3.44 ± 0.50
L29	8.58 ± 2.49	9.25 ± 1.77	7.62 ± 1.47
L30	7.75 ± 0.32	7.65 ± 0.56	7.77 ± 0.48

(b) Agreement between sample weights

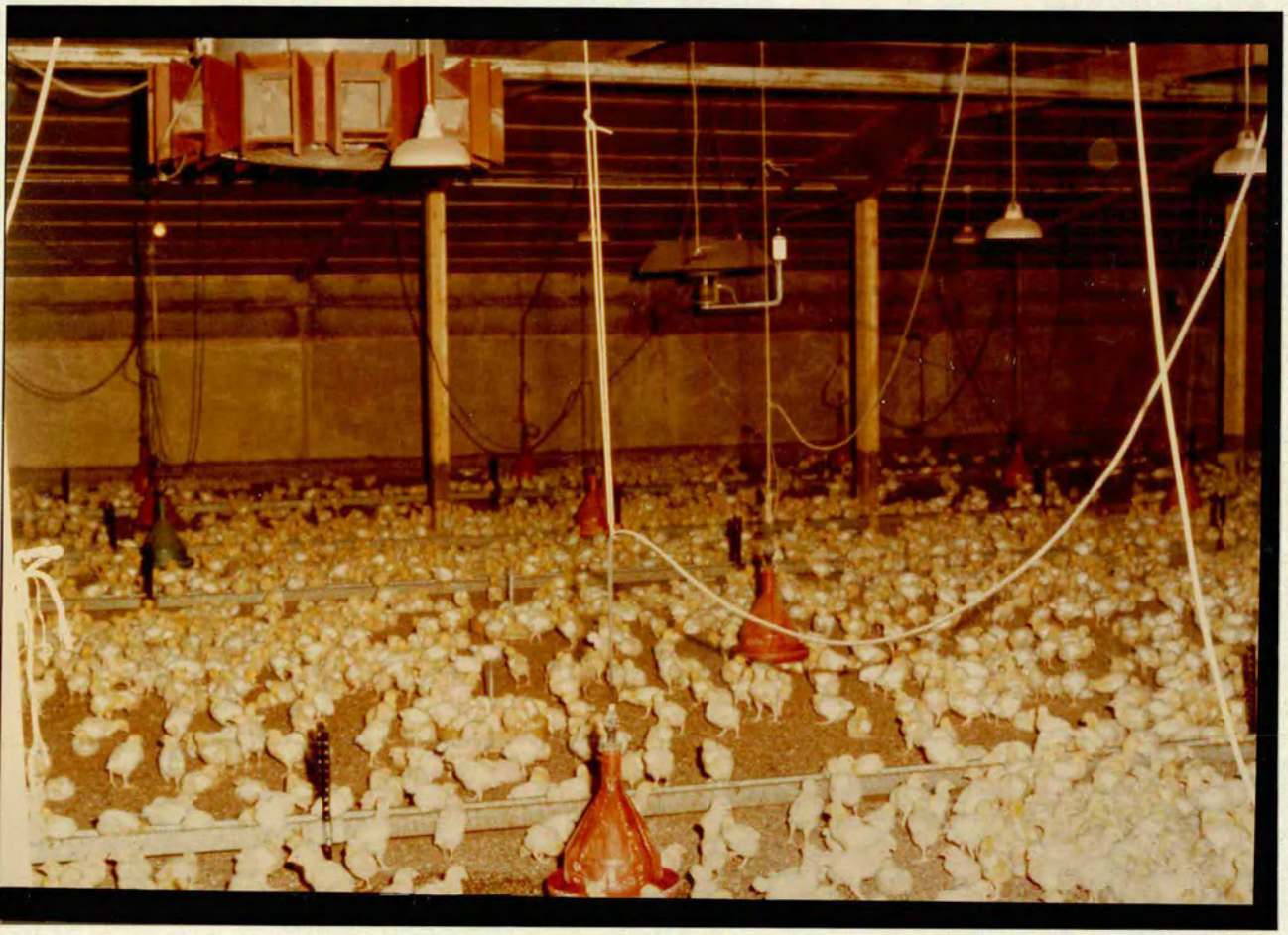
Litter No.	F-value	Probability (2, 12 degrees of freedom
L27	1.48	not significant 10%
L28	0.75	not significant 10%
L29	0.86	not significant 10%
L30	0.10	not significant 10%

The major differences between sample may be attributed to inadequate mixing andloss of viability of the salmonellas during the storage period. The latter is indicated in the results of the 1g sub-sampling where there is no error due to re-weighing at the time of testing.

As there was a health risk when weighing contaminated material the 1g sample size was adapted for all experiments in which litters were inoculated in the laboratory.

Photograph 4.1

The Interior of a Modern Broiler House.



2 The Variability of Litter within Comparable Sampling Stations in Broiler Houses (Experiment 4.2)

The variability of litters both within a very small area and on comparable sites in three broiler houses were compared. The houses which were on the same unit all contained birds of 36d on the day of sampling.

Experimental Method

Sampling at each station was carried out within the confines of a circle of diameter 50cm at approximately 2m from the walls at each corner of the houses (A, B, D, E). A fifth sampling station was in the centre of the building (station C). At each station five samples were taken by marking a circle of 7cm in diameter and removing all the litter within this area to a depth of 2cm.

All samples were examined 24h after collection the interim storage being at ambient temperatures.

The following parameters were determined:-

1. Total aerobic count at 26°C on N Agar.
2. Total anaerobic count at 26°C on N Agar.
3. pH value.
4. Moisture Content.
5. Inhibition of salmonellas by weighing 1g of each litter sample and inoculating these with 0.1ml of 10^{-4} dilution of S. typhimurium. After incubation at 26°C for 48h the numbers of salmonellas was estimated using the stated MPN-3 method.

Results and Discussion

The visual appearance of the samples collected within each house were uniform, however as can be seen in Table 4.2 there was considerable variation between the samples from the different houses despite the birds being of the same age.

Table 4.2

Summary of Statistical Analysis of Variation between Broiler Houses (raw data detailed in Table A4 (ii) in Appendix 2)

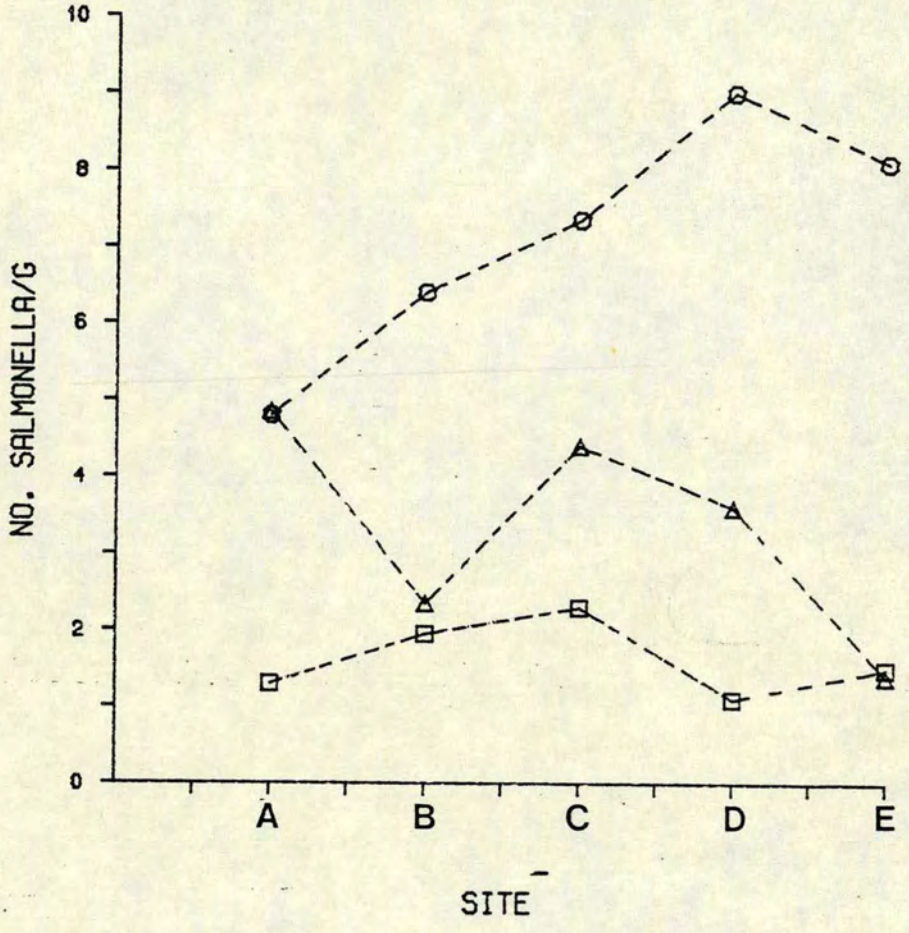
(a) By House

Parameter	F-value of determination of:-			
	House 1	House 2	House 3	All houses
Aerobic count	10.31***	1.09	28.24***	7.04**
Anaerobic count	37.51***	NA	40.84***	5.01**
Number of salmonellas persisting per g	3.75**	11.94***	1.63*	40.08****
pH	42.66***	2.10*	6.04**	27.95***
MC%	11.22***	1.02	2.55**	13.93***

(b) Position in House-all parameters

House number	Sampling Station				
	A	B	C	D	E
1	13.26***	12.00***	9.08**	9.83**	9.54***
2	10.84***	11.81***	11.79***	11.89***	11.43***
3	11.78***	12.39***	12.27***	12.12***	10.89***

(C) PERSISTANCE OF SALMONELLA.



(D) MOISTURE CONTENT (%).

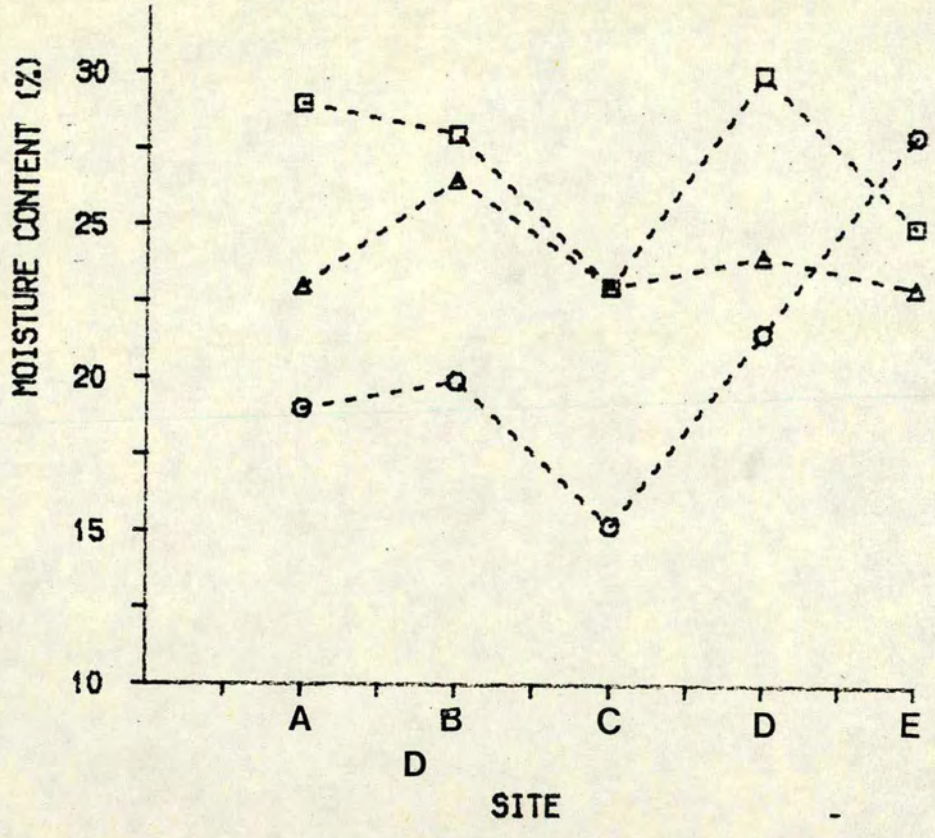
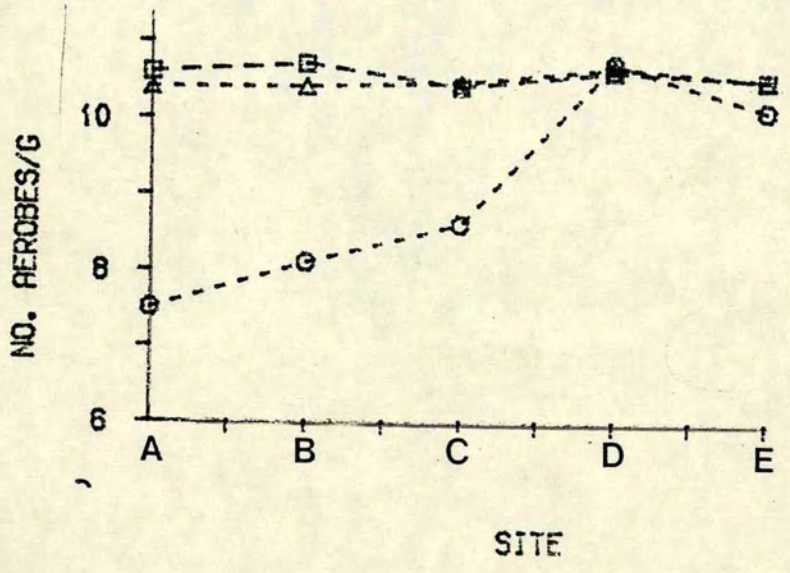


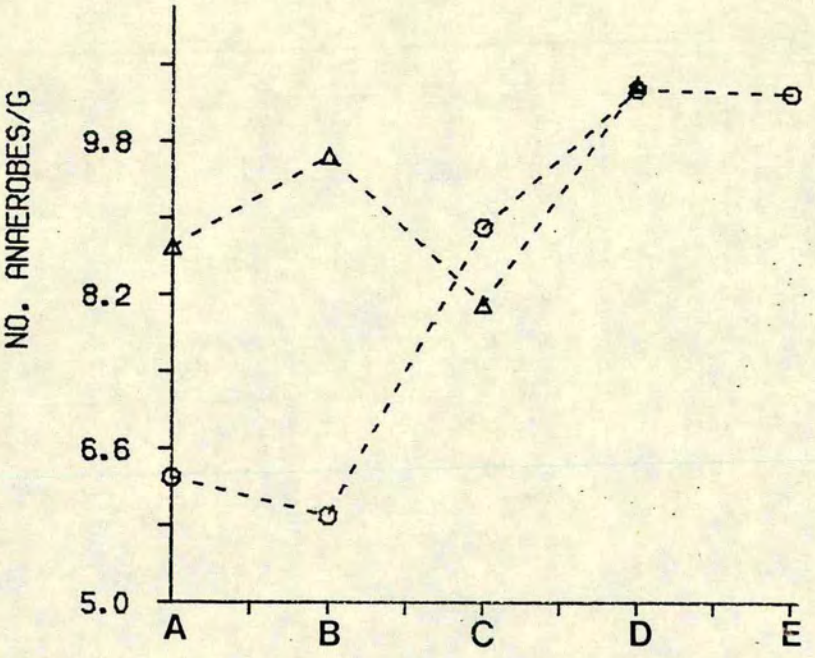
FIG.4.1. THE VARIABILITY OF LITTER AT COMPARABLE SITES WITHIN BROILER HOUSES AT 3 ENTERPRISES.

(KEY. ○ HOUSE 1, △ HOUSE 2, □ HOUSE 3.)

(A) NUMBER OF AEROBIC BACTERIA.



(B) ANAEROBIC BACTERIA.
HOUSES.



There was little uniformity between the parameters from House 1 and 3. Fig. 4.1 shows the mean value for each parameter at each sampling station within each house. As can be seen there is little difference between the mean values for each site. Except for the lower moisture content at station C of all the houses there was no consistent pattern relating the site position and the parameter measured in this experiment.

These results show that within a small area litter is relatively uniform but there is a lack of consistency throughout the whole house. Therefore to obtain a representative sample of litter it is essential to collect a large number of sub-samples from throughout the houses. Unless otherwise indicated at least 50 sub-samples were taken from the total area of each house examined.

C General Discussion

The representative sampling of poultry litter is beset with difficulties. However the value of the results of any investigation is limited by the extent to which the initial sample and subsequent sub-samples represent the bulk of the material. In addition the handling and preparation may cause changes which influence the results of the microbiological examination of the material. To overcome this demur all the litters used in this study were collected immediately prior to the initiation of each examination and on those occasions when it was necessary to store the samples this was never longer than 24h in a cold store at 4°C.

When considering data from experiments of any material such as litter small variations between results may be disregarded however. Consistent changes over a period of time one normally regarded as "real" differences. In many of the experiments in this investigation the numbers of salmonellas were determined on one occasion only, therefore in this context it is important to define the change in numbers which will be regarded as representing inhibition or multiplication.

Many workers consider that a 1 $\log_{10}$ i.e.10-fold difference in numbers of bacteria represent a significant change (Parker, 1970; Berkowtz et al., 1974; Coulter & Russell, 1976). However if a series of hypothetical values are taken and the standard errors calculated it can be demonstrated that a 1 $\log_{10}$ difference does not take into account the errors inherent in the sampling and testing procedures. Therefore changes of the order of 2 $\log_{10}$ could be considered as a more realistic value to represent a significant change worthy of being termed inhibition or multiplication.

The degree of inhibition is also related to the numbers of salmonellas inoculated into the material. To take into account this factor when appropriate the level of salmonellas at the end of the incubation period will be expressed as % survivors as determined in the following equation:

$$\% \text{ survivors} = \frac{\text{Number of salmonellas recovered}}{\text{Number of salmonellas inoculated}} \times 100$$

When the results in this form are interpreted in terms of inhibition or multiplication a difference of 2 $\log_{10}$ is equivalent to a 50% change in percentage of survivors. Therefore the results are intepretated as in Table 4.3.

Table 4.3

The Change in bacterial numbers which are considered as representing multiplication or inhibition

Changes in numbers ($\log_{10}$)	% survivors	% kill	Interpretation
+2	150	-	Growth
0	100	-	No change
-2	50	99.9	Inhibition

Prior to calculation all the results are subject to the same magnitude of error, so the factor of error can be disregarded in the computation and the resultant percentage used as an absolute value not subject to margins of error.

SUMMARY

1. One gram sub-samples were chosen as the sample size for all experimental work as statistically there was no advantage in using a larger weight of material.
2. There is considerable variation between sites in a house therefore unless specified all litter samples are
   (a) Taken from the surface layer to a depth of 2cm.

(b) At least 50 sites were sampled per house.

(c) All experiments were set up within 24h of collection of the litter, storage prior to use being at 4°C.

3. The following criteria were taken to represent a significant change in numbers.

(a) A sustained trend in magnitude of a parameter will be considered as significant.

(b) A change of more than $\pm$ 2 $\log_{10}$ or $\pm$ 50% change in numbers will be considered as multiplication or inhibition as appropriate.

CHAPTER V

THE PERSISTENCE OF SALMONELLAS IN RAW INGREDIENTS OF POULTRY LITTER

THE PERSISTENCE OF SALMONELLAS IN RAW INGREDIENTS OF POULTRY LITTER

A Introduction

Poultry litter is a mixture of basal inert material and the faeces of the chicks which as the result of bacterial fermentation develops into a dry friable material.

The attributes of the ideal basal material have been defined by Reed & McCartney (1970) as:

1. Light in weight
2. High absorbent
3. Dries quickly
4. Particle size larger than 2.39mm
5. Soft and compressible
6. Low thermal conductivity
7. Adsorbs minimum moisture from a humid atmosphere

In addition such a material must be easily available locally and inexpensive.

Although a large number of materials have been used none combine all the above factors. Among the materials used worldwide have been wood shavings, wood chips, sawdust, chopped, pulverised or milled straw, shredded newspaper, peat, spent mushroom compost, bracken, pulverised bark, rice hulls, peanut hulls, ground corn cobs, clay, sand, polystyrene residues, plastic offcuts and chips.

In Scotland there is a fairly large forestry industry and so wood products are readily available. Consequently the majority of poultry units in the area served by the East of Scotland College of

Agriculture use wood shavings or sawdust as the base for their litter.

The chemical analysis of wood products used for litter as reported in the literature have a range of 0.1-26.6mg/g of nitrogen, 1.6-2.8mg/g crude fat, 46.4-55.1mg/g carbohydrate, 40-41mg/g acid detergent fibre, 240-300mg/g lignin, 1.8-1.9mg/g phosphate, 22.5mg/g crude protein and 27.6mg/g crude fibre. The pH levels have been reported from pH 5.1 to pH 7.0 and the moisture content from 20% to 50.8% (Table A5 (i) in Appendix 2).

During the rearing period the proportion of faeces to basal material changes continuously for as the bird becomes older the weight of faeces deposited per day increases. Therefore when the chicks are first placed there is approximately 285g of basal material per bird, by 6w it is estimated that each bird will have deposited 1.26kg (wet weight) of faeces (Anon., 1977b). Bacterial action is responsible for the breakdown of the faeces so that there is no obvious increase in the total volume of the litter. The heat produced by the activity provides warmth so aiding the drying process and providing the birds with a warm insulated floor.

Chicken faeces contain a wide range of chemicals (Table A5 (ii) in Appendix 2) the most important being uric acid which is the major nitrogenous excretion. This uric acid is excreted in a colloidal form possibly to aid the water balance of the bird (Young & Dreger, 1933; Sykes, 1971).

The composition of the diet can influence the moisture content of the faeces, for instance high levels of sodium (Hijikuro, 1976;

Milner & Featherstone, 1976), potassium (Hijikuro, 1976) or chloride (Milner & Featherstone, 1976) increase the moisture content of the faeces which may result in the caking of the surfaces (Milner & Featherstone, 1976; Rummels, 1980). Similarly the moisture content of the faeces per se will influence the moisture content of the litter (Arafa et al., 1979). In the same way a high level of protein in the feed will result in an increased amount of feathers on the litter but this can also be a characteristic of some breeds (Twining et al., 1976).

In this section on the persistence of salmonellas in poultry litter the two basic materials, wood products and faeces were examined separately to ascertain the relative importance of these two components.

It was possible to obtain the faeces of broiler birds as the birds used for feed trials at the Poultry Unit of the East of Scotland College of Agriculture are housed in cages. It was therefore possible to collect raw faeces from the cleaning belts and to identify the cage from which the excreta originated.

Wood products were obtained from small sawmills in Perthshire and Angus where it was possible to verify the species of tree being sawn. In the locality of Edinburgh the sawmills bulked all their waste wood and so samples of these contain a mixture of wood of unknown origin.

B Materials

1. Faeces from Broiler Birds housed in Cages

The birds from which faeces were obtained were 56d old birds

housed in cages at the density of 5 birds/cage. The details of the feedstuffs are detailed in Table 5.1.

Table 5.1

Chemical Analysis of diets fed to broilers on Feed Trial at Easter Howgate Poultry Unit (source Nutrition Chemistry Department: East of Scotland College of Agriculture)

Diet Number	DM %	Crude Protein	Fibre	Ether Extract	Ash	Nitrogen free extract	Metabolisable Energy (MJ/kg DM)
A	90.5	184	31	40	68	675	14.0
B	90.1	186	32	41	68	671	14.0
C	88.2	193	39	39	71	655	13.8
D	89.3	196	37	47	76	642	13.9
E	90.8	203	36	54	75	629	14.1
F	91.4	225	41	47	78	606	13.9

Approximately 50g of faeces were removed from under each cage, the cage number being noted.

2. Wood Products

Sawdust and wood shavings were obtained from sawmills who were cutting individual or could identify the woods in mixtures of green woods. The wood species collected were oak, sycamore, spruce and pine and mixtures of oak/beech, spruce/pine and spruce/pine/larch.

C Experimental Work

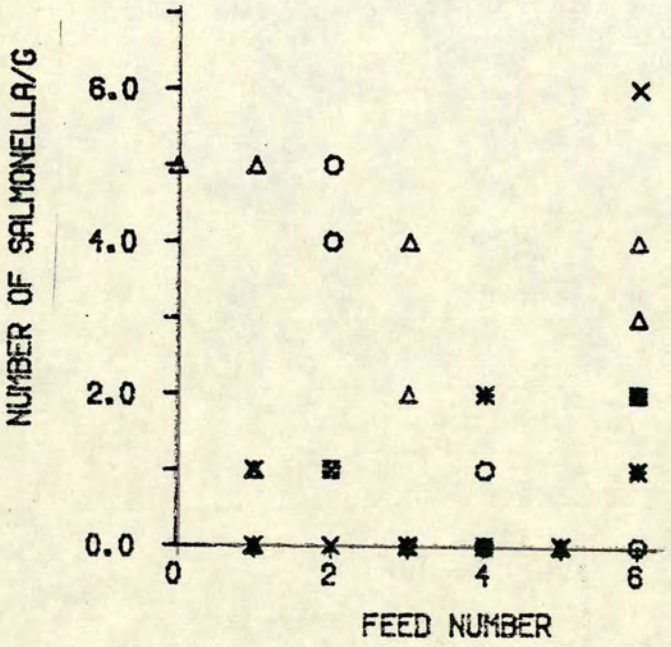
(1) The Persistence of Salmonella typhimurium in the Faeces of Broilers housed in Cages (Experiment 5.1)

Ten grams of each of the faeces samples were weighed into a sterile 60ml plastic vial within 4h of collection from the broiler

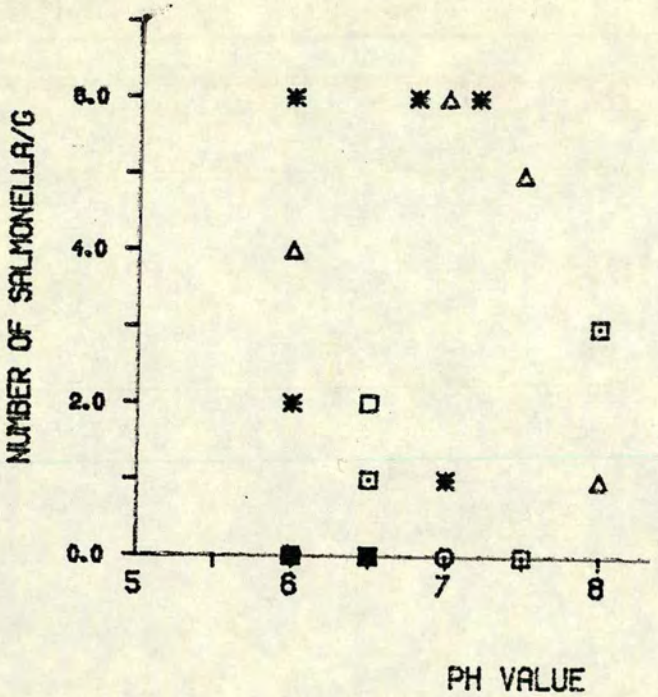
FIG. 5.1. SCATTER DIAGRAMS OF THE RELATIONSHIP OF THE FEEDSTUFFS AND THE PERSISTENCE OF SALMONELLAS IN THE CORRESPONDING FAECES.

(KEY. ○ FEED 1, △ FEED 2, □ FEED 3, ✱ FEED 4, × FEED 5, ◇ FEED 6).

(A) THE RELATIONSHIP OF FEEDSTUFF AND NUMBERS OF SALMONELLAS RECOVERED AFTER 2D.



(B) SCATTER DIAGRAM OF SURVIVAL OF SALMONELLA AND PH OF FAECES.



house and 1ml of a 10^{-3} dilution of an overnight culture of S. typhimurium incorporated. The vials were stored at 26°C for 2d.

The large number of samples to be examined necessitated the use of the MPN-1 method, the initial dilution being 90ml of Ringer's solution to 10g sample. Decimal dilutions up to 10^{-6} were prepared and 1ml of each dilution added to 5ml of LICNR broth. Ten ml of the 10^{-1} dilution was added to 10ml double strength LICNR broth in order to detect the presence of salmonellas if present at less than 1 salmonellas/g.

Results and Discussion

Scatter diagrams of the raw data in Table A5 (iii) in Appendix 2 show that only the faeces of birds consuming Feed 1 inhibited salmonellas, the faeces of all other birds showed no relationship between the persistence of the salmonellas in the faeces and the corresponding feed stuff (Fig. 5.1a). The data presented in Fig. 5.1b show that the difference in the persistence of salmonellas was not related to the pH value of the faeces. reference to the chemical analysis and the formulations of the feedstuffs does not explain these differences.

It was not considered worthwhile undertaking a feed trial specifically to elucidate these points.

2. The Growth of Salmonella typhimurium in Wood Products (Experiment 5.2)

The ability of wood from known species of trees was tested to

determine whether they varied in their ability to support the growth of salmonellas.

Experimental Details

One gram quantities of the sawdusts and wood shavings were weighed into 60ml vials and 0.1ml of a 10^{-4} dilution of an overnight culture of S. typhimurium added. The treated samples were stored at 26°C for 2d when the number of salmonellas were estimated by the MPN-3 method. This experiment was repeated using samples of the wood products which had been heat treated at 121°C for 15 mins.

The pH value and moisture content of the materials were determined at the commencement of the experiment.

Results and Discussion

Only the oak/beech mixture inhibited salmonellas but as the oak wood alone did not have this effect so it must be concluded that the beech wood may have an inhibitory effect. When the experiment was repeated these results were confirmed. (Table 5.2)

Table 5.2

Persistence of Salmonella typhimurium in Sawdust and Woodshavings of known origin

Species of Wood	Number of salmonellas/g (av. 2 results)		MC%	pH value
	No treatment	Autoclaved		
Oak	4.13	1.62	23.08	4.75
Sycamore	6.87	1.18	25.57	4.45
Oak/beech	1.65	>1.00	39.47	3.71
Spruce 1	5.01	4.38	44.62	8.40
Spruce 2	4.82	>1.00	56.90	8.65
Larch (sawdust)	4.77	>1.00	42.38	8.50
Larch (shaving)	5.34	2.38	47.10	7.90
Pine	5.23	2.36	51.17	7.50
Spruce/pine	4.01	1.63	33.30	5.35
Spruce/larch/pine	5.01	1.36	38.40	6.00

Unfortunately it was not possible to examine beech wood separately as it was not available again at the sawmills. It is noteworthy that the oak/beech mixture was the most acidic of the woods tested (pH 3.75) but with this exception the pH did not appear to be related to the persistence of salmonellas (R^2 = 6.2%). Similarly there was no relationship between the growth of salmonellas and the moisture content (R^2 =0) but on the other hand there appeared to be a relationship between the pH value of the wood and the moisture content, the damper woods having a more alkaline pH value although the correlationship was not statistically significant (R^2 = 79.9%).

Heat treatment of the wood products rendered them more inhibitory than the corresponding raw products, although the effect varied between the wood species. This is probably due to the release of phenols and other chemicals by the heating process.

These results indicate that the wood shavings and sawdust are unlikely to be inhibitory to salmonellas and in this context the new litter can be considered to be an inert basal material.

D General Discussion

The sawdust and wood shavings per se in general do not appear to play any part in the inhibitory effect of the litter. In contrast 45.8% of the samples of faeces of mature birds after storage for as little as 2d were able to totally inhibit salmonellas and a further 35.4% of faeces samples inhibited salmonella to a less degree. These result are similar to Gilbert (1954) who showed

an inhibtory factor in faeces but also did not obtain consistent results.

These observations would appear to suggest that the wood products merely act as an inert basal material and the faeces provide both the nutrients and the bacteria responsible for the fermentation process modifying this mixture into litter. As all the original products of the litter are acidic while the litter is alkaline it is evident that there must considerable changes in the constituent species of bacteria during this fermentation. On this basis it could be anticipated that there will be a time-lag between the deposition of faeces on the basal material and the onset of inhibition of salmonella by the litter.

SUMMARY

1. With the exception of beech wood the wood products examined were not inhibitory to salmonellas. The low pH of the beech wood was probably responsible for the inhibition.
2. Heating the wood products rendered all except one sample of spruce wood inhibitory.
3. The moisture content of wood products ranged from 23.08 to 56.90% and pH values from pH 3.75 to 8.50.
4. Fresh poultry faeces vary in their capacity to inhibit salmonellas.
5. This inhibition could not be related to the differences in food composition.
6. The wood products act as an inert base and the faeces provide the medium for bacterial growth.

CHAPTER VI

THE PERSISTENCE OF SALMONELLAS IN POULTRY LITTER

THE PERSISTENCE OF SALMONELLAS IN POULTRY LITTER

A Review of Literature

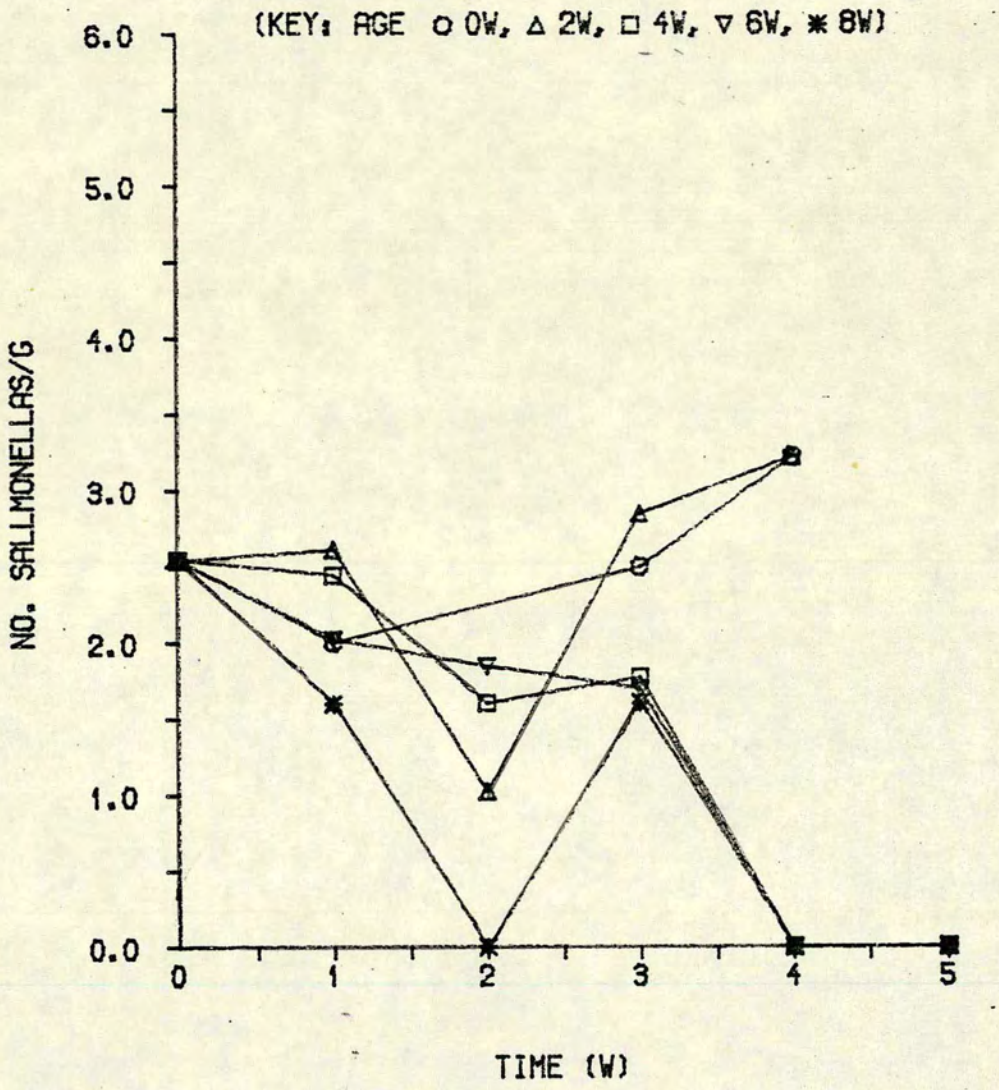
When the method of intensively rearing broilers on deep litter was first developed in America it was common practice to use litter for more than one batch of birds. Disease, including salmonellosis, was a major problem in the early units and this prompted Botts and her co-workers in 1952 to investigate the persistence of salmonellas in litter. In a laboratory experiment they found that *Salmonella pullorum* could survive for 70d in new corn cob litter but only 15d in old litter. This observation has been repeated by many workers (Table 6.1). Unfortunately many workers give little information on the criteria they use to decide the difference between a new litter and old litter. However it can be seen that there is a marked inhibition of salmonellas by the older litter and consequently the ability of old litter to be self-sterilising with respect to pathogens is generally accepted by the poultry industry. (Table 6.1)

Table 6.1

The Survival of Salmonellas in Litter cited in Literature

Author	Salmonella Serotype	Base Material	Time of Survival (d) in New Litter	Old Litter
Botts *et al.* (1952)	*S. pullorum*	Corn cob	70	15
	S. gallinarium		63	-
Tucker (1967)	*S. pullorum*) *S. gallinarium*)	Wood shavings	77	21
	S. thompson	Wood shavings	42-140	35
Olesiuk *et al.* (1969)	*S. typhimurium*	Wood shavings	49-63	14-28
Snoeyenbos (1967)	*S. typhimurium*	Wood shavings	77	14
Duff *et al.* (1974)	*S. typhimurium*	Wood shavings	-	14
Dougherty (1976)	*S. simsburg* *S. sont paul* *S. senftenkerg*	Wood shavings	42-56	24-42

FIG. 6.1. THE PERSISTENCE OF SALMONELLA AGONA IN LITTERS OF VARIOUS AGES.



B Experimental Work

1. The Persistence of Salmonella agona in Poultry Litter of Various Ages (Experiment 6.1)

An experiment was carried out to establish the pattern of persistence of salmonellas in litter of various ages. S. agona was selected for inoculation in this experiment as this was the serotype indigenous to the enterprise from which the litter was collected.

Experimental Details

Litter was collected from broiler houses with birds of 3d, 2w, 4w, 6w and 8w of age (L31-35). At the laboratory 5 sub-samples of 1g of each litter were weighed into glass MacCartney bottles, inoculated with 1ml of 10^{-4} dilution of S. agona and stored at 26°C. At weekly intervals the number of salmonellas in one sub-sample was determined. The experiment was continued for 5 weeks.

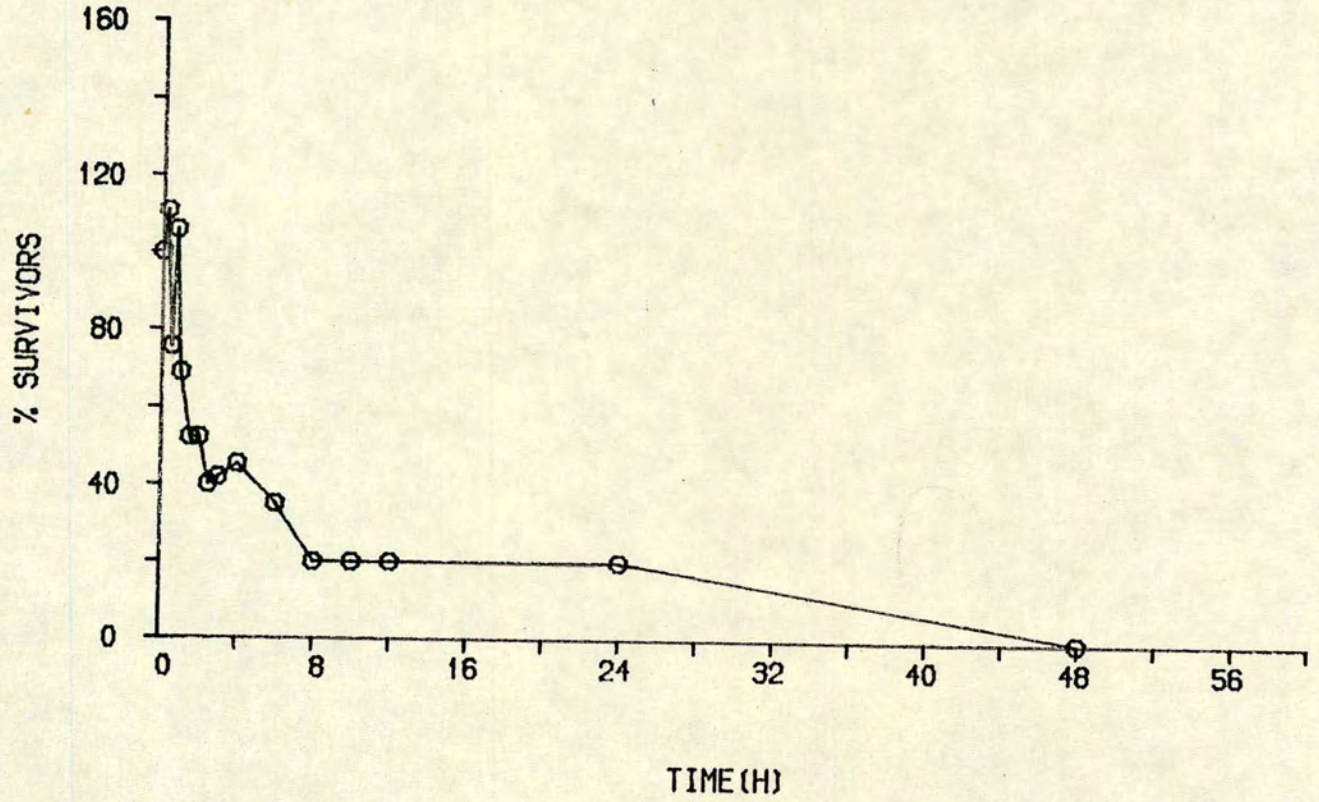
Results and Discussion

During the first two weeks the number of salmonellas decreased in all the litters. By the third week the number of salmonellas in the litter of 3d and 2w old had increased significantly and remained at this high level for the duration of the experiment. In contrast in the older litter the numbers continued to decrease, no salmonellas being detected after 4 weeks (Fig. 6.1).

2. The Rate of Decline in Numbers of Salmonella typhimurium in Mature Litter in 5 days (Experiment 6.2)

The results of the previous experiment confirms that mature litters are more inhibitory to salmonellas than immature litters.

FIG 6.2. PERSISTENCE OF SALMONELLAS IM MATURE LITTER INOCULATED IN THE LABORATORY.



This experiment will determine the rate of decline in numbers of salmonellas in a mature litter in the laboratory.

Experimental Details

Twenty, 1g quantities of a 5-6w litter (L37) were weighed into 25ml glass vials and each inoculated with 0.1ml of an overnight culture of S. typhimurium. After thoroughly shaking by hand the vials were stored at 26°C and at intervals one sub-sample was taken and the number of salmonellas determined by the stated MPN-3 method.

Results and Discussion

The numbers of S. typhimurium was reduced by 50% of the original inoculum within 3h and by 80% in 8h. The contamination then remained at 20% for the next 16h when it further reduced to 5% of the original numbers (Fig. 6.2).

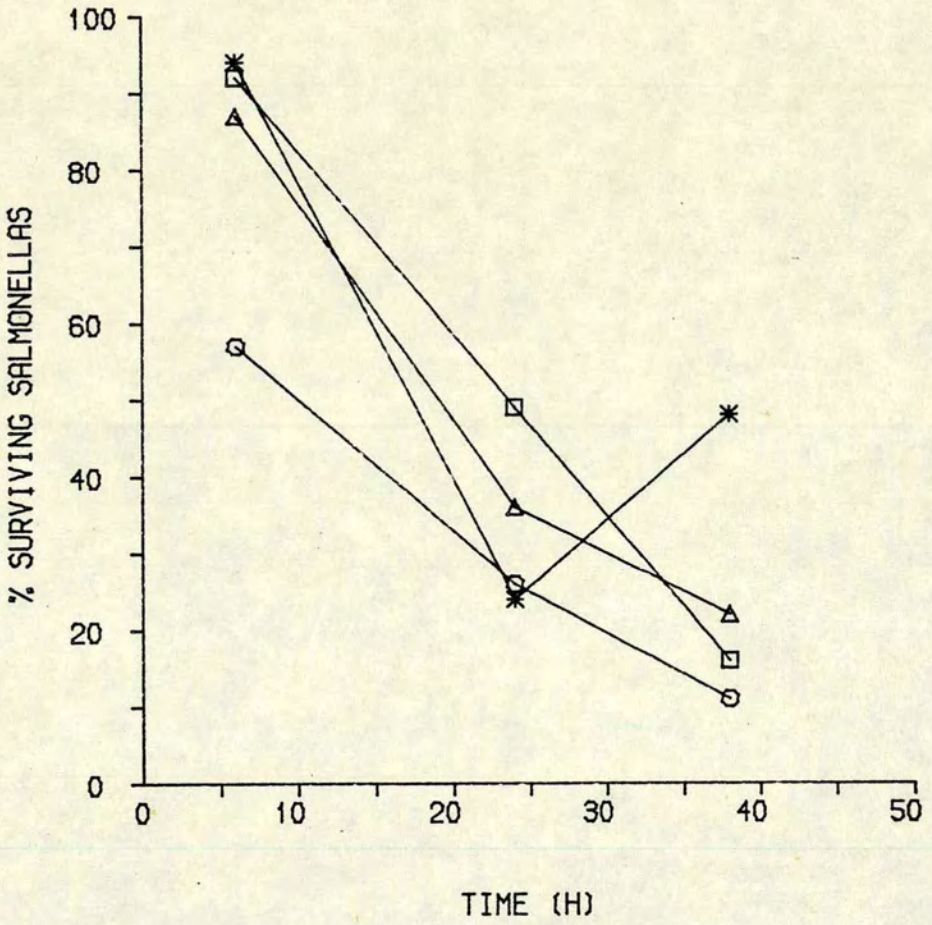
These results indicate that immediately after deposition on the litter the number of salmonellas will be reduced rapidly but a small proportion of the bacteria continue to persist so providing a constant focus of infection.

3. Quantifying the Nature of Inhibition of Salmonella typhimurium in Poultry Litter (Experiment 6.3)

As already discussed Tucker (1967) and Turnbull & Snoeyenbos (1973) agree that the low moisture contents are partly responsible for the inhibition of salmonellas in litter. Turnbull & Snoeyenbos (1973) also suggested that the alkaline pH of the mature litter resulting from the production of ammonia was a parameter involved. In addition

FIG. 6.3. THE EFFECT OF REMOVING INHIBITORY FACTORS FROM POULTRY LITTER ON THE PERSISTANCE OF SALMONELLAS.

(KEY: ○ UNTREATED, △ "COARSE EXTRACT", □ FILTERED, ✳ SEIZT STERILISED



Tucker (1967) suggest that bacterial antagonism could also be involved. Therefore an experiment was carried out in which some parameters were eliminated in order to indicate the relative importance of these factors.

Experimental Details

Litter of 21d, 36d and 56d (L 37-39) subjected to the following treatments.

1. 1g quantities of litter - untreated.
2. 20ml of 10^{-1} dilution in Ringer's solution filtered through sterile butter muslin.
3. 20ml of (2) filtered through Whatman No. 1 filter paper.
4. 20ml of (2) sterilised by Seitz Filtration.
5. Control - 20ml of sterile BP Water.

0.2ml of an overnight culture of S. typhimurium was added to each of the above and the aliquots incubated at 26°C for 2d. The number of salmonellas was determined by the stated MPN-3 method.

This experiment was repeated on three occasions using litters of the same ages but from different houses.

Results and Discussions

These results confirm that older litters are more inhibitory to salmonellas than younger litters (Table A 6(i) in Appendix 2). For simplicity the mean value for each treatment, at each sampling time were calculated and are shown graphically in Fig. 6.3. From this diagram it can be seen that when the litter was diluted the adverse effect of water activity was removed, the resultant

solution was slightly less inhibitory than the original litter. Removal of the solid material resulted in a solution which initially had less inhibitory action but by 24h the effect was very similar to that of the diluted litter. On the other hand when the bacteria were removed by filtration this solution is very much less inhibitory than the other treatments as at 48h, 70% of the salmonellas survived in contrast only 3% survived in the diluted litter, 15% of a conventionally filtered solution and 11% in untreated litter. These results confirm that the inhibition of salmonellas involve a number of factors, in particular it does appear that bacterial activity is involved in addition to physico-chemical factors.

4. The Transfer of Bacteria between Litters and the Persistence of Salmonella typhimurium in the Resultant "Litters" (Experiment 6.4)

The foregoing experiment showed that bacterial antagonism is one of the factors involved in the inhibition of salmonellas in poultry litter. This being so it should be possible to transfer the activity of an inhibitory litter to a non-inhibitory litter by transferring the bacteria from the former litter to the latter. The following experiment was designed to test this hypothesis.

Experimental Details

Two litters were selected one of 44d which have proved to be very inhibitory to salmonellas (L36) while the other of 14d (L37) had shown not to inhibit salmonellas. For clarity they will be referred

to as Litter H and Litter L respectively in this account.

Ten gram of each litter was added to 100ml of Ringer's solution and allowed to remain for 2h at room temperature with occasional shaking. The liquid was expressed by filtration through a double layer of butter muslin, and then filtered by passing through Whatman filter paper No. 1 and 50ml of the filtrate centrifuged for 15mins at 2,000 rpm. The deposited cells were then washed 6 times in Ringer's solution and re-suspended in 10ml N broth to a solution with a turbidity reading 25 nephelometer units.

The following treatments were set up by atomizing 2ml of each of the bacterial mixtures on 20g of litter spread over a 9cm petri dish (ie equivalent to 10^{-1} dilution).

(a) Litter H as collected (H)

(b) Litter L as collected (L)

(c) Litter H autoclaved, with bacteria from Litter H (HAH)

(d) Litter H autoclaved, with bacteria from Litter L (HAL)

(e) Litter L autoclaved, with bacteria from Litter H (LAH)

(f) Litter L autoclaved, with bacteria from Litter L (LAL)

(g) Litter H autoclaved (HA)

(h) Litter L autocalved (LA)

Seven, 1g quantities of each treatment were stored at 26°C. At 0d, 2d, 4d, 6d, 8d and 10d one replicate of each treatment was inoculated with 0.1ml of 10^{-4} dilution of an overnight culture of S. typhimurium and the numbers of salmonellas in these "litters"

enumerated after a further 2d incubation at 26°C using the stated MPN-3 method. At day 10 the remaining sub-samples were autoclaved and then incubated with S. typhimurium and the number of salmonellas surviving determined as above.

Results and Discussion

The results obtained after inoculation on day 0 confirmed the inhibitory nature of Litter H and the manner in which Litter L favours the growth of salmonellas, the anticipated effect being shown in the inoculated "litter". Gradually all the litter became inhibitory irrespective of whether the microflora was derived from Litter H or Litter L although the "Litters" inoculated with microflora of Litter H become inhibitory within a shorter period of time than the litters treated with microflora L. In contrast the autoclaved litter did not become inhibitory until after 6th day but never attained the same degree of inhibition as the other "litters". (Table 6.2)

Table 6.2

The Transfer of Bacteria between Litters and the effect of the Resultant Litters on the Inhibition of Salmonella typhimurium (*see experimental details for explanation of code; <1.00 present in 2g)

Treatment*	Salmonellas persisting in litter+ inoculated at:- 0	2	4	6	8	10	Autoclaved Litter 10d	Final pH
H	1.63	3.36	1.36	2.00	2.00	<1.00	2.00	9
L	7.66	5.60	1.00	<1.00	1.36	0.60	6.38	7.5
LAL	7.38	5.36	0.60	<1.00	1.36	<1.00	3.97	8.5
LAH	7.38	1.36	0.60	<1.00	<1.00	<1.00	8.38	8.0
HAL	4.63	3.36	<1.00	1.36	<1.00	<1.00	8.66	8.0
HAH	3.66	1.00	<1.00	<1.00	<1.00	<1.00	7.38	7.5
HA	2.18	3.36	6.18	1.63	4.04	3.72	8.66	7.5
LA	>8.04	5.78	5.32	3.04	4.46	1.36	8.18	7.5

Therefore it can be seen that the inhibitory effect can be transferred from one litter to another by transferring the micro-flora. However in time the incubation condition in the laboratory would appear to favour those bacteria in both litters which inhibit salmonellas. The involvement of bacteria in the inhibitory function of these "litters" is confirmed by the loss of this attribute on rendering the litter sterile by autoclaving. As the pH value of all the "litters" except the control Litter H had values less than pH 8.5 this factor would not appear to be involved in these effects demonstrated in this experiment.

C General Discussion

It cannot be assumed that the mode of activity of salmonellas in litter within the poultry house is exactly the same as that determined in laboratory experiments. However the experiments in this section show that as the litter matures it becomes increasingly inhibitory to salmonellas thus confirming the circumstantial evidence in the literature. Determination of the rate of decline in numbers of salmonellas show that while the litter inhibits it does not eliminate these pathogenic bacteria. Therefore once contaminated the litter will continue to be a potential source of infection should conditions change so that it loses its ability to inhibit salmonellas. However it would appear that large numbers of salmonellas are required in the litter to cause an out-break of salmonellosis in the birds (Tucker, 1967; Duff et al., 1974) so it would seem unlikely to occur in practice.

It is common practice within the broiler industry to examine litter samples for salmonellas at 3-5 w to determine the infection status of the flock with respect to salmonellas. In the literature there is a divergence of opinion as to the usefulness of this routine testing. This difference may result from the difference of sensitivity of the methods of bacteriological examination coupled with different storage times between collection and laboratory analysis. Evidence, presented here, of the rate of decline in numbers of salmonellas in litter indicates that such a monitoring programme will serve to confirm the presence or absence of infected birds in the flocks but give no indication of the magnitude of the problem.

The data in this section has also confirmed that the inhibition of salmonellas by litter results from interaction of a number of factors of a physico-chemical and bacteriological nature. This mechanism of inhibition will be further examined in later sections of this thesis.

SUMMARY

1. Litter becomes increasing inhibitory during the rearing period of broiler birds.
2. When a 5/6w litter was artificially inoculated with salmonellas the numbers of viable cells decrease rapidly in the first 8h and remain at low numbers for at least 5d.
4. The inhibitory nature of a litter can be transferred to another litter by transferring the microflora but laboratory incubation

favours the antagonistic bacteria in the inoculum.

5. The inhibitory nature of litter is due to a complex interaction of physico-chemical parameters coupled with bacterial activity.

CHAPTER VII

THE INHIBITION OF SALMONELLAS IN LITTER DERIVED FROM COMMERCIAL BROILER HOUSES

THE INHIBITION OF SALMONELLAS IN LITTER DERIVED FROM COMMERCIAL BROILER HOUSES

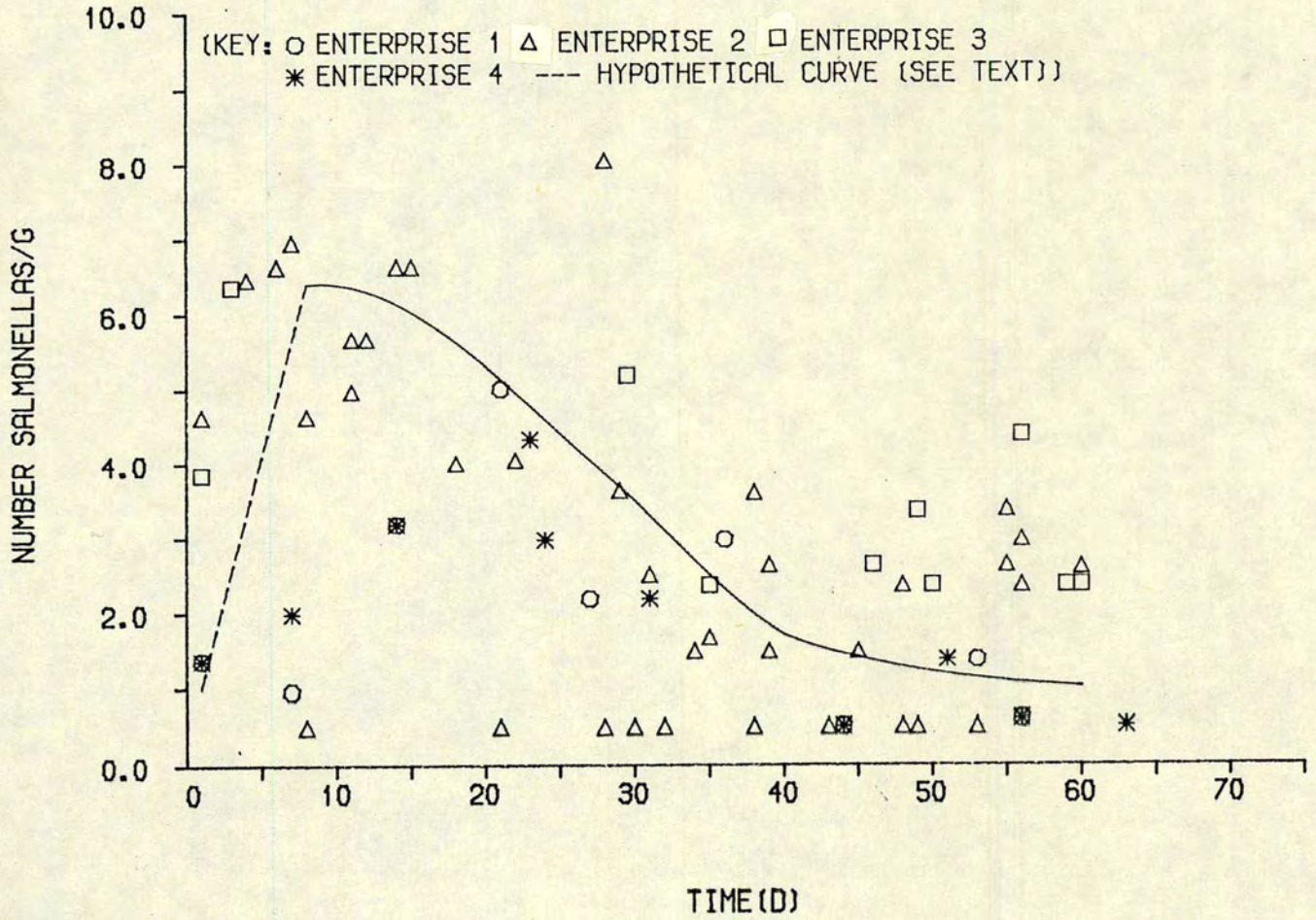
A Introduction

The foregoing sections have examined the variability of litter within and between houses in relation to sampling procedures and have established the ability of some litters to inhibit salmonellas. Before exploring the mechanism of inhibition it is essential to confirm that this phenomenon occurs sufficiently frequently to warrant investigation. Therefore a survey of litter from commercial broiler houses was undertaken, samples being taken from houses at four enterprises representing all stages of rearing.

Samples were also undertaken at three houses containing birds of the same age to determine whether the variations in litter could be explained by factors such as position in the house, house furniture etc. Analysis of results may also indicate these features of the litter which would predispose the establishment or breakdown of the inhibitory action.

It was not possible to obtain litters which have been seeded with salmonellas by birds therefore a laboratory method was used to ascertain changes in the degree of persistence of salmonellas in the litter. In these experiments the litters were inoculated a few hours after collection from the house so reproducing as far as possible the effect that the litter would exert on naturally excreted salmonella. The storage time of two days was chosen as the results of Experiment 6.2 showed that the inhibition was completed within this period of time.

FIG. 7.1. THE PERSISTENCE OF SALMONELLAS IN LITTERS FROM COMMERCIAL POULTRY HOUSES.



B Experimental Work

1. The Persistence of Salmonella typhimurium in Litter from Commercial Broiler Houses after inoculation in the Laboratory (Monitoring Exercise 7.1)

In order to substantiate the claims that poultry litter is inhibitory to salmonellas a survey was undertaken in the east of Scotland in which the litter from houses containing birds of all ages were collected from four enterprises.

Experimental Details

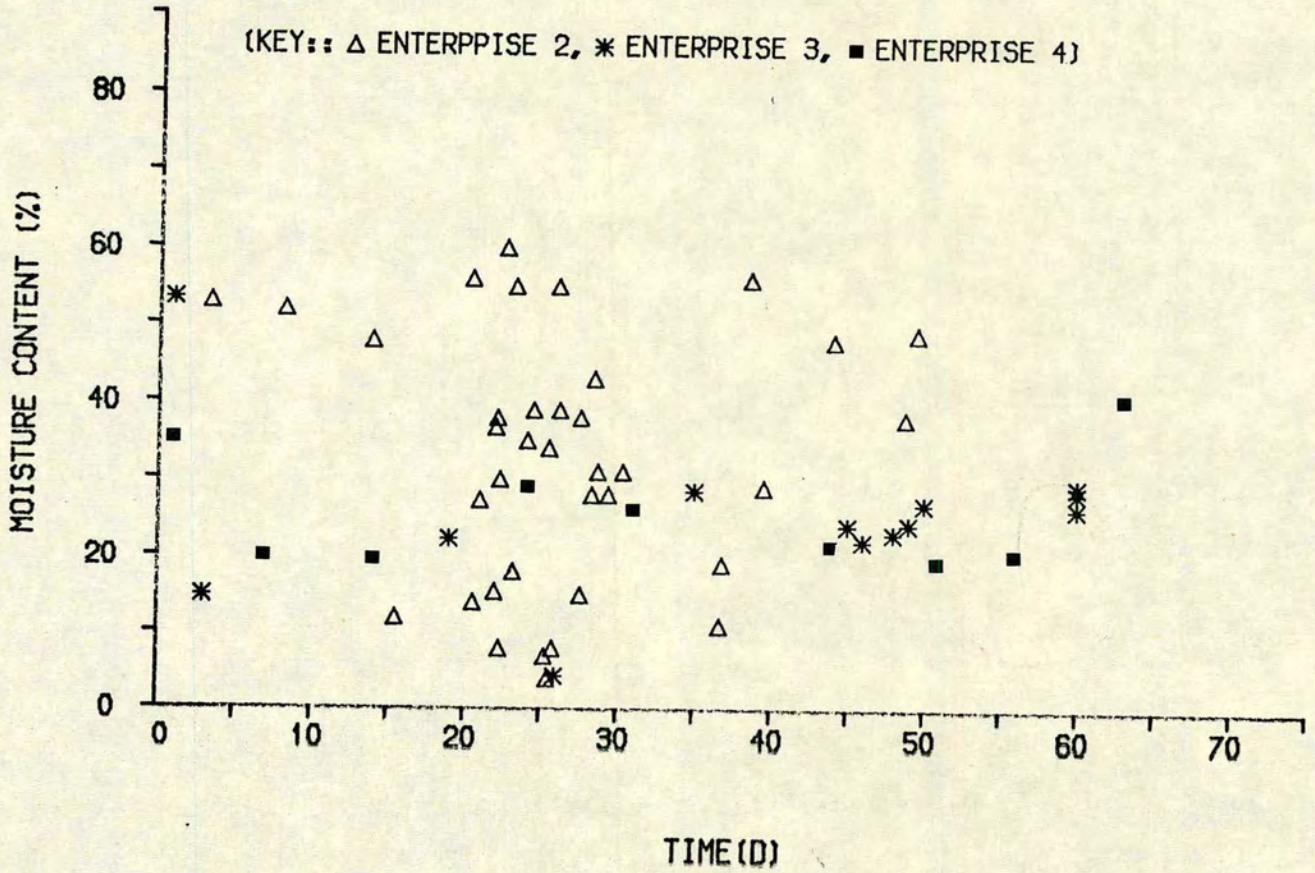
Seventy nine houses owned by four independent companies were visited and samples of the litter collected as described in the General Materials and Methods. Within 8h of sampling 1g quantities of each litter was weighed out and 0.1ml of 10^{-4} dilution of an overnight culture of S. typhimurium added. After storage at 26°C for 48h the number of salmonellas in each sub-sample was determined by the stated MPN-3 method. The moisture content and pH value of the litters were determined on the day of collection.

Concurrently the litters were also examined for the presence of indigenous salmonellas by the method of Morgan-Jones (1982b). S. agona was recovered in three samples and therefore these litters were not included in the final results.

Results and Discussion

The results from this survey confirmed that there is in general a trend for litters to become increasing alkaline and in their ability to inhibit salmonellas. However as can be seen in Fig. 7.1

FIG. 7.2. THE MOISTURE CONTENT (%) OF LITTER FROM COMMERCIAL POULTRY HOUSES.



the picture is complex but three phases can be identified:-

Phase 1: Initially the litter may be either inhibitory or allow salmonellas to proliferate. This is possibly due to the efficiency with which fumigation is undertaken and the differences of woods to absorb and release the fumigant. Unfortunately this survey was undertaken before the determination of the level of formaldehyde was a routine analysis and so it is not possible to confirm this supposition. This effect was not associated with specific enterprises and so could not be related to a particular wood product or managerial practice.

Phase 2 Salmonella multiply in litter

Phase 3: As the litter matures they become increasing inhibitory to salmonellas but this capacity varies greatly between houses.

As the data had a wide spread of values it was not possible to compute a mathematically valid curve. Therefore to interpret these results regression equations and correlation coefficients were determined for intervals which were selected by inspection of the scatter diagram. Examination of the statistical analysis showed that during the period 10 to 35 d there was an increase with time of ability of the litter to inhibit salmonellas but this trend was not statistically significant (R^2 = 34.9%). After 25d all litters inhibited salmonellas to some degree.

The moisture content of the litter did appear to be directly related to time but closer examination showed that from 0 to 15d the litter tends to become damper (R^2 = 21.1%) and then ranged from 20% to 40% moisture for the following 30d (R^2 = 0.0%) after which the moisture content varied greatly (Fig. 7.2).

FIG.7.4

Inter-relationship of pH and growth of *S. typhimurium* in litters inoculated at laboratory.
(pH not adjusted)

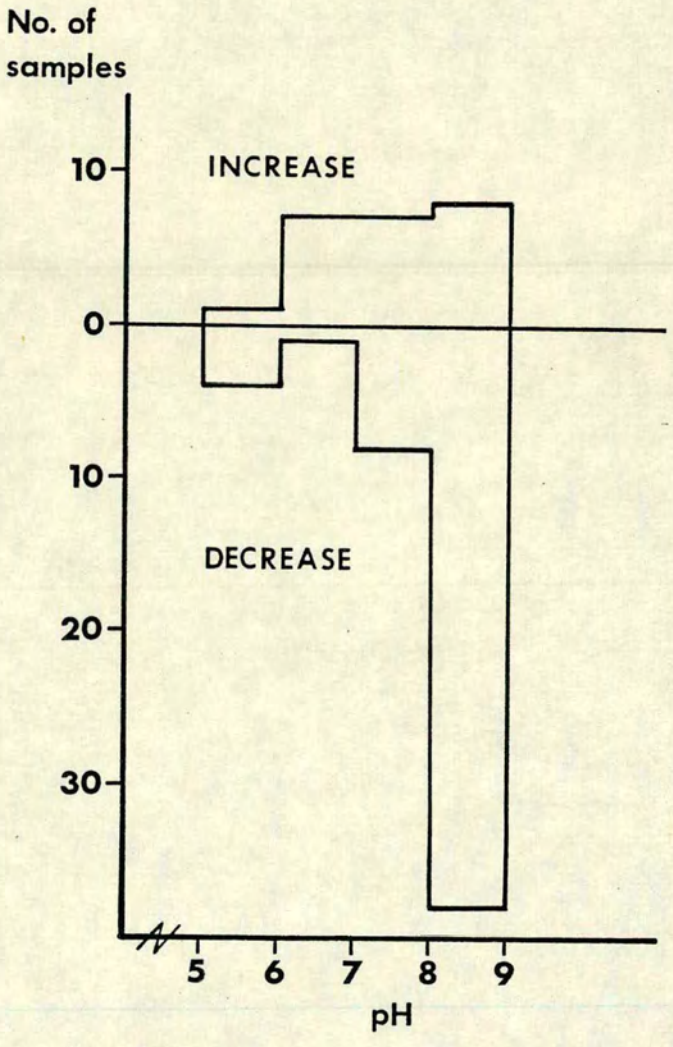
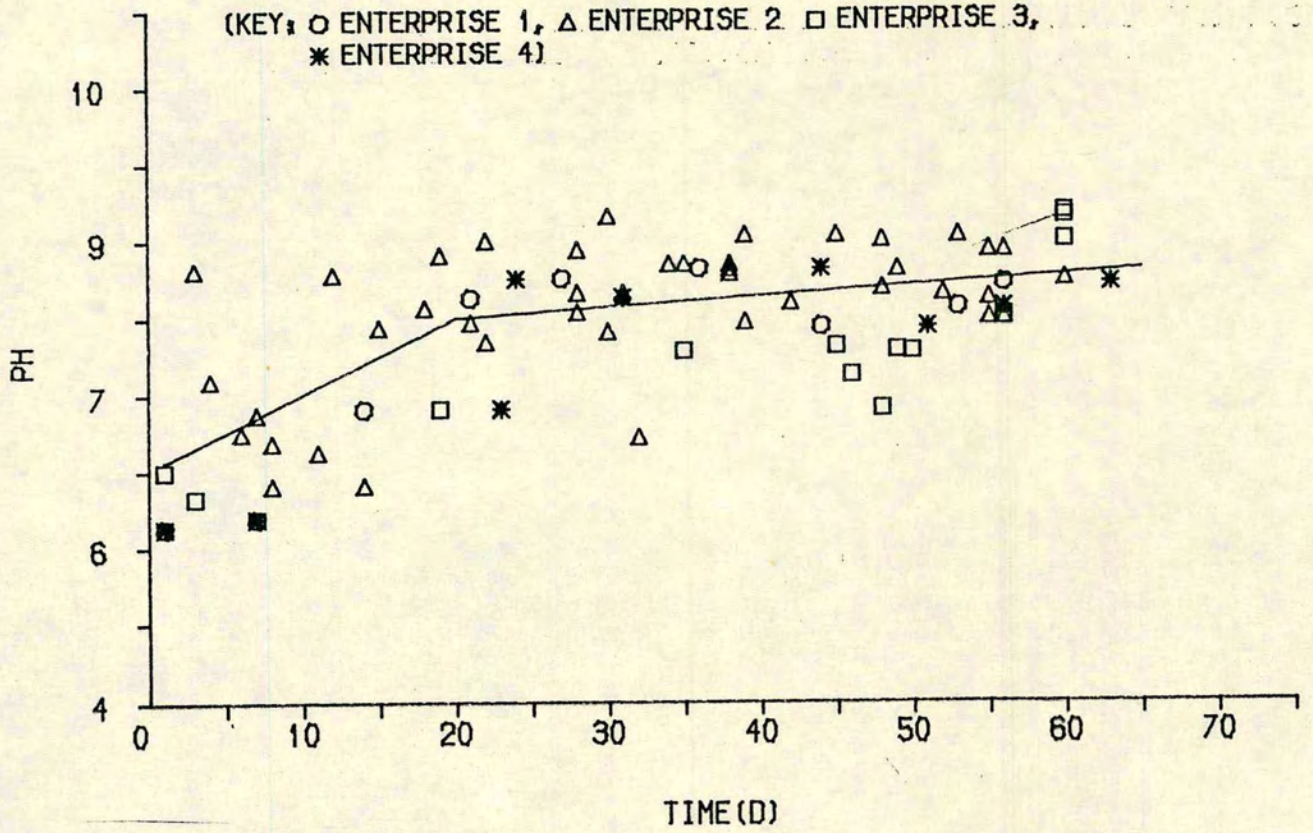


FIG.7.3. THE PH LEVEL IN LITTERS FROM POULTRY HOUSES.



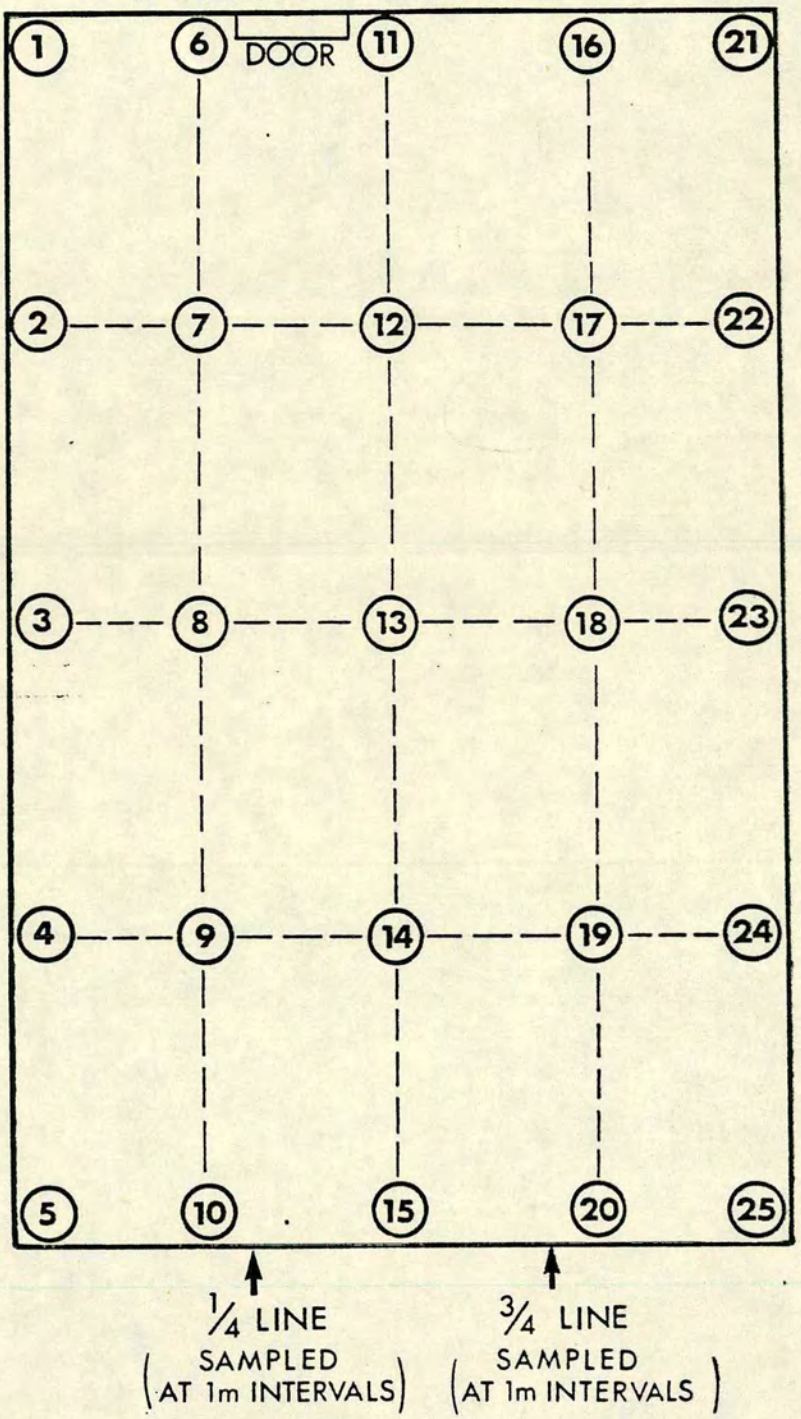
The relationship of a pH value of the litter with age presented the most clear cut picture (Fig. 7.3) in that as the litter becomes older it became increasing alkaline (R^2 = 44.7%). This change appeared to be in two phases, initially between 0 to approximately 30d this change was rapid (R^2 = 63.5%) and later there is little variation of pH value (R^2 = 1.9%). When the regression equation for the critical periods of time were solved and plotted it could be seen that the pH level appears to stabilise between 25 to 35d

When the inter-relationship of the parameters were compared there would appear to be no relationship between the moisture content and pH value or the persistence of salmonellas. In contrast there was a relationship between the inhibition of salmonellas and the pH of the litter, this is illustrated by totalling those samples in which salmonellas increase or decrease for each pH value. Examining the histogram (Fig. 7.4) it can be seen that at pH 8-9 the salmonellas are very likely to be inhibited (71%) but some litters did not have this effect (19%) although the numbers of these salmonellas surviving was always very low.

These results show that the interactions of litters and salmonellas is diverse in nature and it would appear that no direct relationship exists between these three parameters even after mathematically converting the data (Table A7 (iii) in Appendix 2). However this survey confirms the claim that in general terms as litter matures they become increasingly inhibitory to salmonellas. Between 25 and 35d the conditions in the litter appear to stabilise (R^2 of multiple regression = 55.7%). It is noteworthy that this occurs soon

FIG.7.5.

SAMPLING STATIONS TO DETERMINE THE PERSISTANCE OF SALMONELLA IN BROILER HOUSE.



after the chicks have completed their first moult and the diet has been changed from a chick mash to a fattening ration.

2. The Persistence of Salmonella typhimurium in Litter at Various Sites within Broiler Houses under Commercial Conditions (Monitoring Exercise 7.2)

A detailed sampling programme has been undertaken to ascertain the variation in the litter taking into account the sites within the house and the presence of house furniture. These results were aimed at identifying areas where contamination might persist and by comparing the features of inhibitory and non-inhibitory litters with the object of more clearly defining the characteristics of those litters which are responsible for this inhibition.

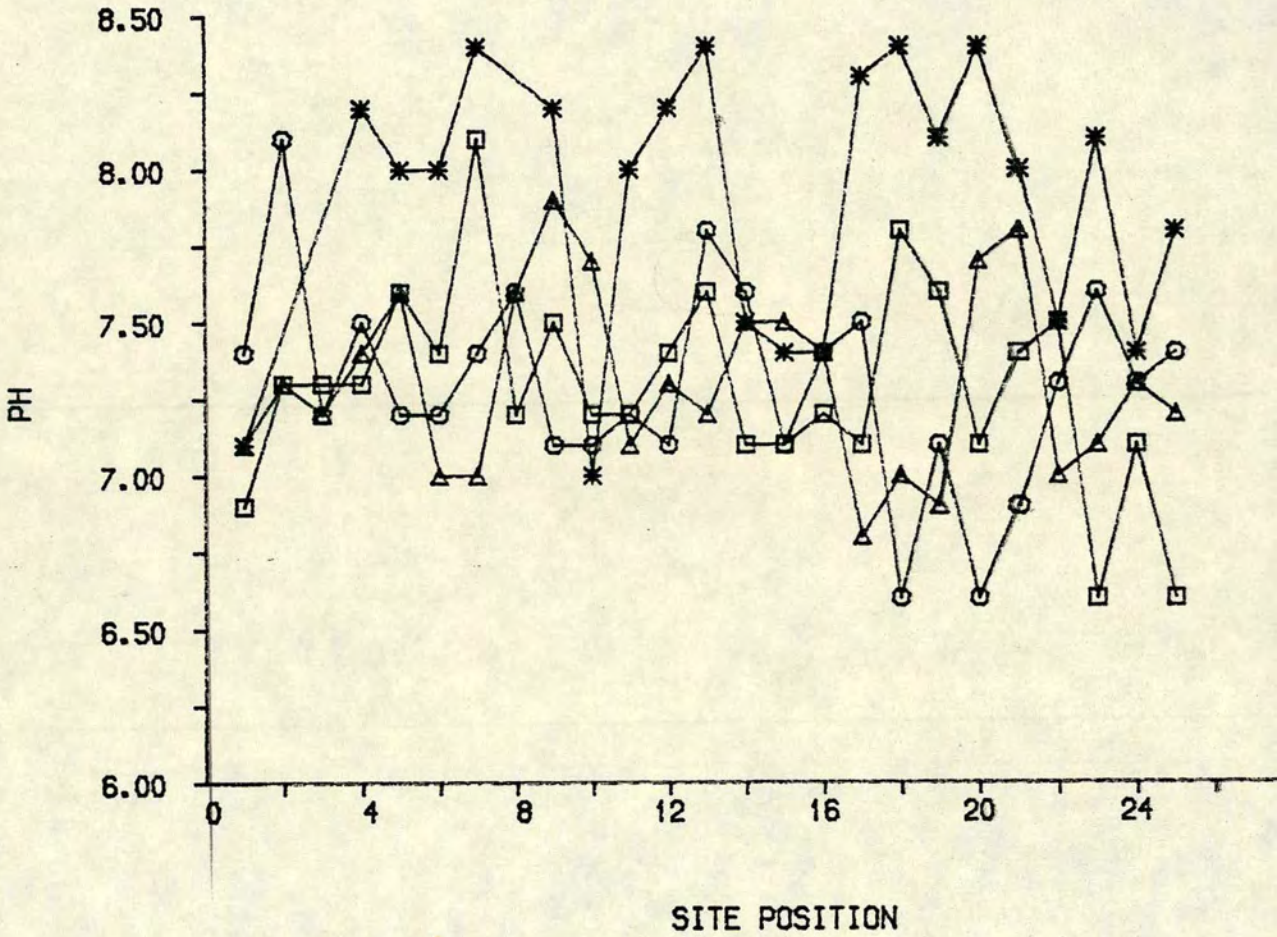
Experimental Details

Three sampling programmes were undertaken viz.:-

1. To determine the variation within the house, the samples being taken at the points (1-25) indicated in Fig. 7.5 (Houses A-D).
2. To determine the influence of the house furniture. Samples were taken at 4m intervals along the quarter-way line (Line A) and three-quarter way line (Line C) in Fig. 7.5 (Houses A, C).
3. To determine the effect of the drying on the litter samples were taken under each brooder and at a central point between each brooder in Fig. 7.5 (Line B) (Houses A-C).

At most sampling points the upper and lower layers of the litter

(C). THE PH VALUE



(B) THE MOISTURE CONTENT(%).

(B) THE MOISTURE CONTENT(%) AT EACH SAMPLING STATION.

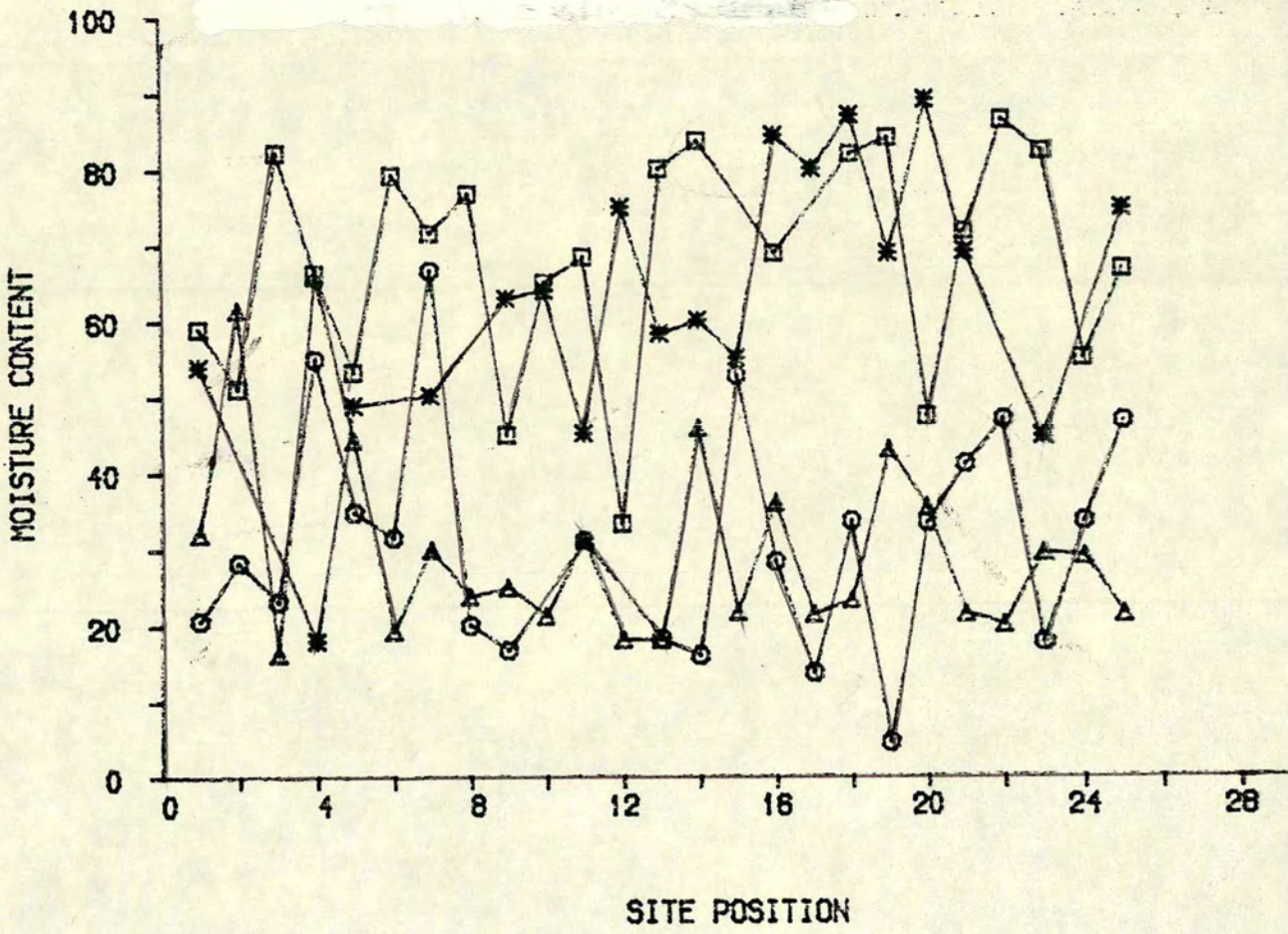
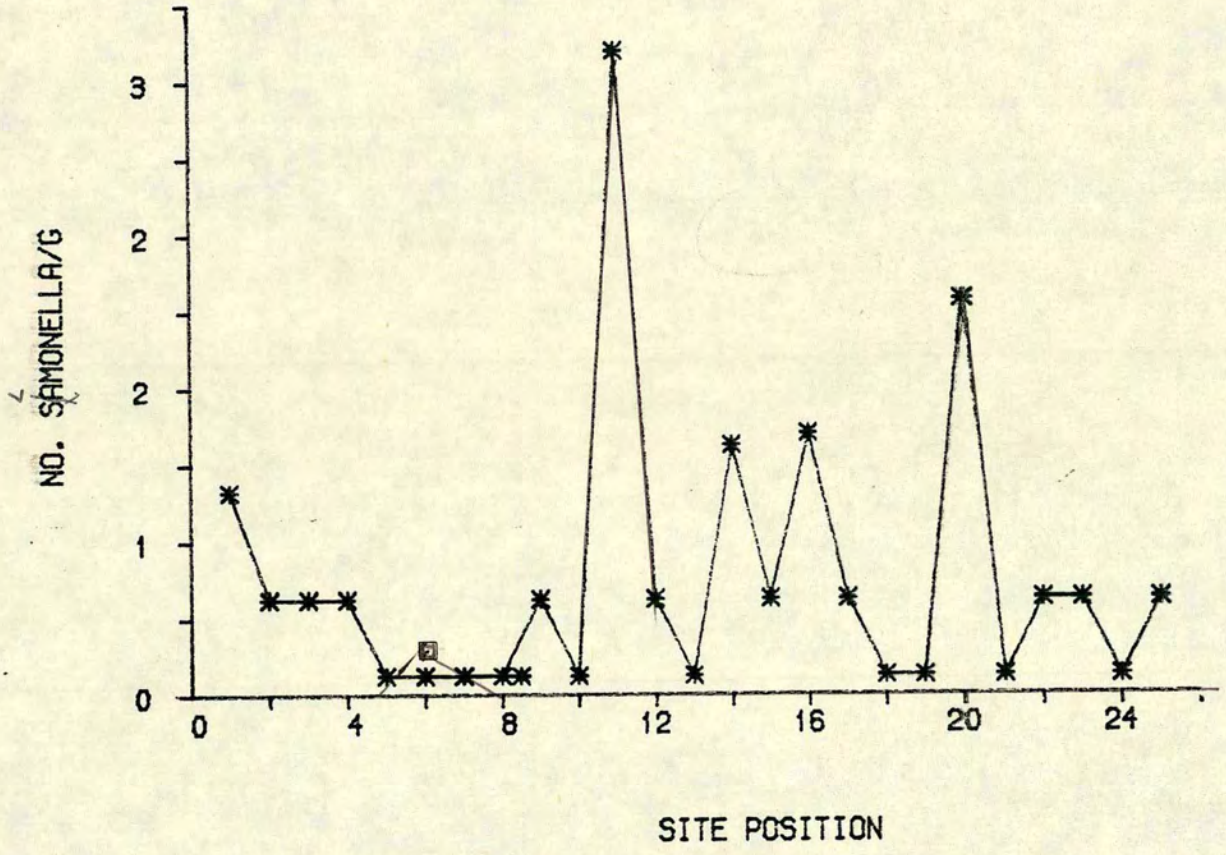


FIG.7.6(A) PERSISTENCE OF SALMONELLAS IN LITTER FROM 25 SITES IN BROILER HOUSE.

(KEY: ○ HOUSE A, △ HOUSE B, □ HOUSE C, ✱ HOUSE D)

(A) PERSISTENCE OF SALMONELLAS.



were sampled separately. the persistence of salmonellas in the litters was determined by inoculating 1g of the litter with 0.1ml of a 10^{-4} dilution of an overnight culture of S. typhimurium which was adjusted to a reading of 25 nephelometer units. After incubation for 2d at 26°C the number of salmonellas was determined using the stated MPN-3 method.

The pH value and moisture content was determined within 24h of collection.

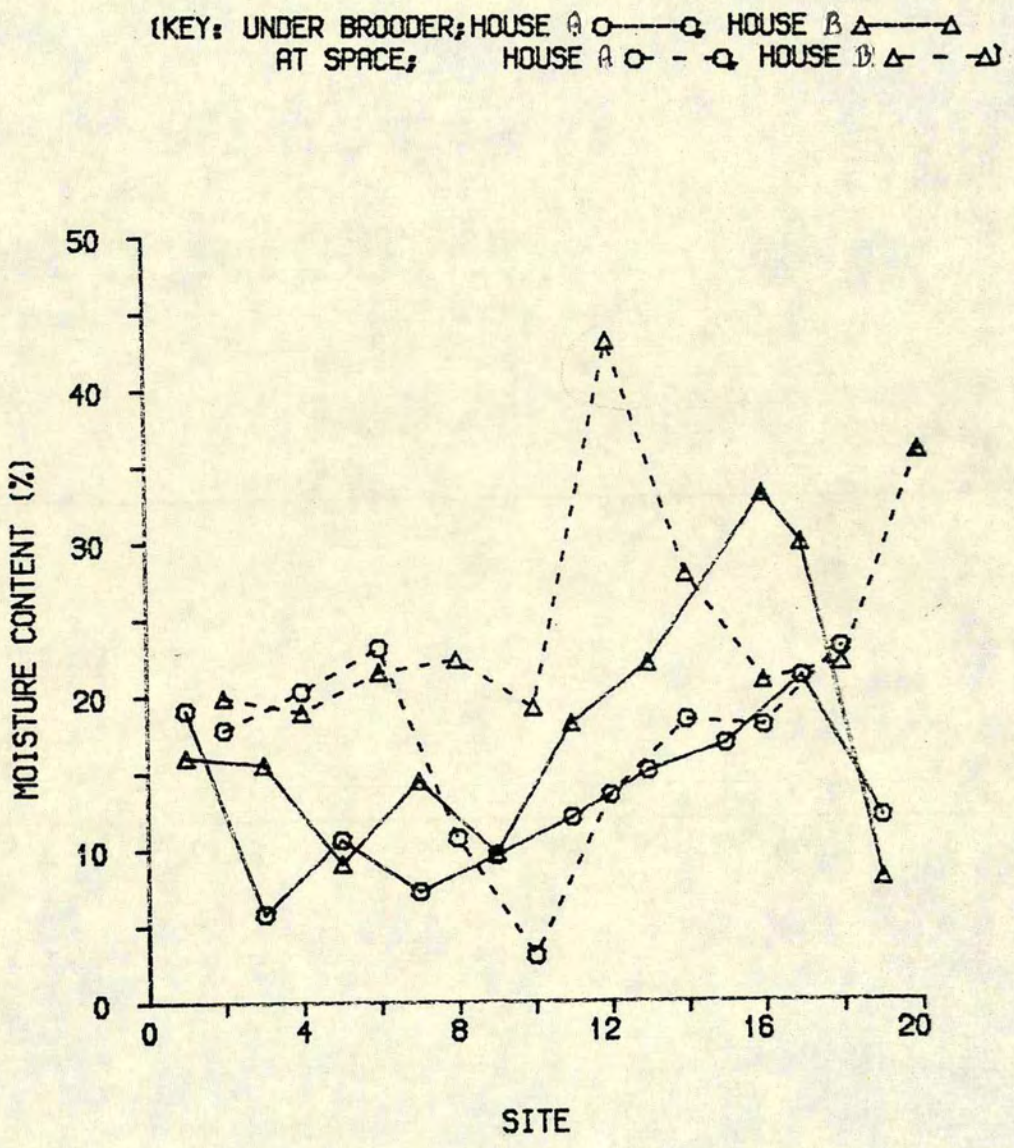
Results and Discussion

All the samples of litter from House B inhibited salmonellas while only a few litters from House A and House C allowed these bacteria to multiply but in House D there was growth in 10 of 25 samples (Fig. 7.6a). The pH value of the litters from House D was slightly higher than the other three houses and the moisture content of both House C and D was higher than Houses A and B(Fig. 7.6b).

There was no correlation between the sites of origin within the house and the values of the parameter examined except for an increase of pH value at the centre of lines 1A and 1C in House 1 which corresponded to a greater degree of inhibiton of salmonellas than samples from the mid-line (Fig. 7.6c). This effect was not found in the other houses. The position of the house furniture did not appear to be related to variations within the litter.

The gas heaters are normally along the central line of the house so it can be assumed that the litter directly below them will be drier than the remaining material. At these sites there was a slight relationship between the moisture content and the pH value

FIG.7.7. THE MOISTURE CONTENT OF LITTER ON THE CENTRAL LINE OF BROILER HOUSE.



(R^2 = 52.3%) and on average there was 3% less moisture in the areas below the brooders than in the spaces between the brooders (Fig. 7.7).

In House B where the litters were inhibitory there was a slight reduction of moisture content at the extremity of the house and additionally the lower layer was moister than the upper layer (significant at 2.5% level). In contrast in House C, 13 of 20 samples collected along the central line allowed the survival of salmonellas, these sites of origin being equally distributed between the brooder areas and the spaces between. The lack of relationship between any of the parameters and locations in each house confirm the lack of spatial relationship previously reported from this laboratory (Morgan-Jones, 1980).

When the data from those sites which were inhibitory to salmonellas are compared to the litter which allowed salmonellas to persist little difference was seen in pH value but the non-inhibitory litter had a mean moisture content of 60·99%, 16% more than the inhibitory litter. As will be seen later this represents a difference in water activity of Aw = 0.99 to Aw = 0.94 so in these cases it is possible that the dryness of the litter is in part responsible for this inhibition but as the range of moisture content in each group of results overlaps there must be other factors involved.

C General Discussion

The fate of salmonellas deposited on poultry litter varies greatly depending on the age of the particular litter. Samples examined from a wide range of poultry houses showed that there are

three phases during which the effect of the litter on the persistence of salmonellas differs. These may be summarised as follows:-

Phase 1: During the first few days the salmonellas may be inhibited by the residual formaldehyde.

Phase 2: A period during which salmonellas survive and may multiply.

Phase 3: A mature litter in which salmonellas are inhibited but which there is considerably variation in the degree of inhibition.

As seen in Fig. 7.1 it is very difficult to define a time scale for each phase as they appear to overlap as follows:-

Phase 1 - 0-10d

Phase 2 - 3-35d

Phase 3 - 22d to end of rearing period

In the literature it is postulated that mature litters are inhibitory to salmonellas. These results confirm that older litters do have this ability. The following question arises "What is a mature litter?" One definition which could be applied is "a litter is mature when it is able to inhibit salmonellas", but this definition does not take into account older litters which have not acquired or lost inhibitory characteristics, and could not be applied in practice by the industry. Moisture content and pH values are values which are easily measured and so a definition involving these factors would be useful in practice. At this juncture it would appear that the moisture content plays little part in the inhibition of salmonellas. However the pH rises initially and then after 25-35d the level stays fairly constant between pH 7.5 to pH 9.0. Taking these parameters

into consideration it could be said that "a litter is mature when it has supported birds for more than 30d and has a pH value over pH 7.5".

The second question which is propounded is "What is an inhibitory litter and how does this differ from a non-inhibitory litter?". Analysis of all the results in this section present a very confused picture. Comparison of the mean value of the moisture content and the pH value of those litters able to inhibit salmonellas and so allowing the survival of salmonellas showed that the inhibitory litters are drier than comparable non-inhibitory litters in the same situation. However it was noted that some litters with apparently equal moisture contents and pH values could be inhibitory in one situation and non-inhibitory in another situation. This would suggest that other factors are involved in this phenomenon.

As these experiments were of a preliminary nature it is not surprising that the results are equivocal but they do confirm that the mode of inhibition is complex in nature. This being established the following sections will explore each parameter in order to suggest the mechanism of this inhibition and to relate these findings to commercial practice.

SUMMARY

1. The data obtained from experimentation with litters from commercial houses show that initially the residual formaldehyde in the litter inhibits salmonellas then follows a period when salmonellas may persist and after 30d most litters inhibit salmonellas.

2. The exact time at which litters become inhibitory vary from house to house under the same management.

3. The pH level of the litter appears to be major but not the sole factor in the inhibitory action.

CHAPTER VIII

THE PERSISTENCE OF SALMONELLAS IN LITTER DURING 0-7d OF OCCUPATION

THE PERSISTENCE OF SALMONELLAS IN LITTER DURING 0-7d OF OCCUPATION

A Introduction

On commercial poultry farms it is normal practice to fumigate the houses with formaldehyde 2-5d before the chicks are placed in order to disinfect the complete environment. Fumigation is achieved by heating paraformaldehyde granules, adding formaldehyde solution to potassium permanganate or spraying with an aerosol of a solution of formalin (see General Materials and Methods, 2E). Under neutral or alkaline conditions formaldehyde reacts with cellulose to form hemiocatoles and acetol or fumol linkages (Walker, 1964). However it can be expected that the levels of formaldehyde incorporated in the litter under farm conditions will be at a low level as even when wood was treated with formaldehyde in a kiln at 120°C only 40% of the gas was adsorbed (Storm, 1959).

The bacteriocidal properties of formaldehyde is occassionally used in nest boxes when flakes or tablets of paraformaldehyde are added to the litter (Anon., 1974). When the hen sits on the nest to lay her body heat releases the gas which fumigates the nest material onto which the egg is to be deposited.

When Velso et al. (1974) applied the same principle to floor litter they found that the addition of 3% paraformaldehyde to the litter reduced the bacterial count by 10% together with a reduction of pH for the first 3w over a pH range of 7.22-8.08.

The only report of the use of formalin solution in poultry litter was by Williams (1980) who carried out four trials applying

formalin solution to sawdust at 2%, 4% and 6% level on a volume basis. The solution was mixed into the wood shavings by turning the litter one to three times. The ability of the litter to inhibit salmonellas was determined by culturing litter samples taken after the placements artificially infected with a nalidixic acid resistant strain of Salmonella typhimurium. In the longterm 2% was ineffective, the 4% level was inhibitory on some occasions and 6% level was always effective. Unfortunately the levels of formaldehyde in the litter was not assayed but the residual formaldehyde did not appear to interfere with the isolation of salmonellas. The result of the treatments at 2% and 4% level are interesting as the 2% level was able to inhibit salmonellas for at least 7d and 4% level for at least 12d.

In the literature there is no information on the concentrations of formaldehyde in sawdust or wood shavings in poultry houses and the rate at which these substances are eliminated. In the hospital situation Alder et al. (1971) showed that treated woollen blankets could retain formaldehyde at a bacteriocidal level for 7 months if packed but 5w when in regular use. Dickson et al. (1970) similarly found that a level of 0.96% formaldehyde in woollen blankets was reduced to 0.81% when rinsed and 0.64% when aired. Both of these workers attributed the anti-bacterial action of treated blankets to slow evolution of formaldehyde vapour.

Formaldehyde can be inactivated with ammonia by the following reaction:-

$$6CH_2O + 4NH_3 \rightarrow C_6H_{12}N_4 + 6H_2O + 81 \text{ kcal}$$

(Walker, 1964)

It is therefore probable that the ammonia in the chick faeces will also serve to reduce the level of formaldehyde in the litter.

Formaldehyde can also be assimilated into cell wall material and oxidised by some groups of micro-organisms at least five metabolic pathways being described for this reaction (Antony, 1982). To date no workers have reported isolating bacteria possessing formaldehydrogenase from poultry faeces or litter (Mead, 1983). As this enzyme is associated with methanotrophs the presence of methane gas in the caecum demonstrated by Shrimpton (1963) would suggest that bacteria with this activity may be present in the gut. However these bacteria require specialist methods and techniques for isolation so it is very possible that no workers have attempted to isolate these bacteria. If present in the litter they could contribute to the overall reduction of formaldehyde in the litter.

In the previous chapter it was concluded that there were three phases of persistence of salmonellas in litter, the first of these stages is possibly due to the bacteriostatic activity of formaldehyde absorbed on the wood product used for the litter which will affect the salmonellas deposited as well as retard the establishment of the indigenous microflora of the litter. The length of time for which this effect persists appears to be very variable therefore the manner in which formaldehyde disappears from the litter will be now be investigated.

B Materials

1. Sawdust and Wood Shavings in Known Origin

(a) The woods of known species are as described in the Materials

of Chapter 5.

(b) Sawdust and wood shavings of mixed residues of unknown species of trees were collected from either:-

(i) The Dalhousie Sawmills, Loanhead, Midlothian

(ii) The normal supply used by D.B. Marshalls (Newbridge) Ltd., and Hamish Morrison Ltd., Earlston.

C Experimental Work

1. The Effect of Fumigation in the Laboratory on the Growth of Salmonella typhimurium in Wood Products of Known Origin (Experiment 8.1)

In order to ascertain the variations in the ability of the woods of different species of trees to adsorb formaldehyde wood shavings and sawdust of representative species were fumigated in the laboratory.

Fumigated wood products were also heat treated by autoclaving to determine the stability of the association of the formaldehyde and the wood.

Experimental Details

One gram quantities of the wood products of known origin were treated as follows:-

1. No treatment (Control)
2. Heated at 121°C for 15 min.
3. Fumigated in the laboratory (Chapter 2 E [a])
4. Fumigated in the laboratory and 24h later heat treated as (2).

After 24h storage at room temperature the sub-samples were inoculated with 0·1ml of 10^{-4} dilution of S. typhimurium and incubated at 26°C for 2d.

Results and Discussion

Except for the beech wood salmonellas will multiply in all the species of wood tested confirming the results in the Experiment 5.2. After fumigation no salmonellas grew but after heat treatment the results were more variable (Table 8.1) but in all cases these samples proved to be more inhibitory than the corresponding control samples. This inhibition could be explained by the release of

Table 8.1

The Persistence of S. typhimurium in Wood Products treated with formaldehyde and heat treated (raw data in Table A8 (i) Appendix 2)

	% salmonellas surviving in material treated:-				Level of formaldehyde (mg/g) after fumigation
	Not fumigated		Fumigated		
Wood Production	Control	Heat Treated	Control	Heat Treated	Control (x 100)
Oak	181.5	81.0	0	0	7
Sycamore	319.0	59.0	0	0	2.1
Oak/beech	97.5	0	0	0	7
Spruce 1	233.0	219.0	0	0	4.3
Spruce 2	269.0	0	0	0	8
Larch/sawdust	259.0	0	0	0	1.4
Larch/shavings	266.0	119.0	0	0	4
Pine	248.5	118.0	0	0	5.5
Spruce/pine	219.0	81.5	0	0	5.4
Sprice/pine/ larch	219.0	68.0	0	0	5.0

aldehyde and phenolic compounds as the result of heating. However there is no statistical correlation between the control and autoclaved samples so heating appears to affect each species in a different manner.

The wood purchased from the sawmill will vary greatly as the species of trees, age of wood, time of harvest, treatment since cutting, heart or sapwood and physical characteristics of the product will not be constant. Therefore it can be expected that the adsorbtion of formaldehyde by the initial litter will vary from batch to batch of wood residues delivered to the farm.

2. The Growth of Salmonellas in Sawdust with a Range of Known Levels of Formaldehyde (Experiment 8.2)

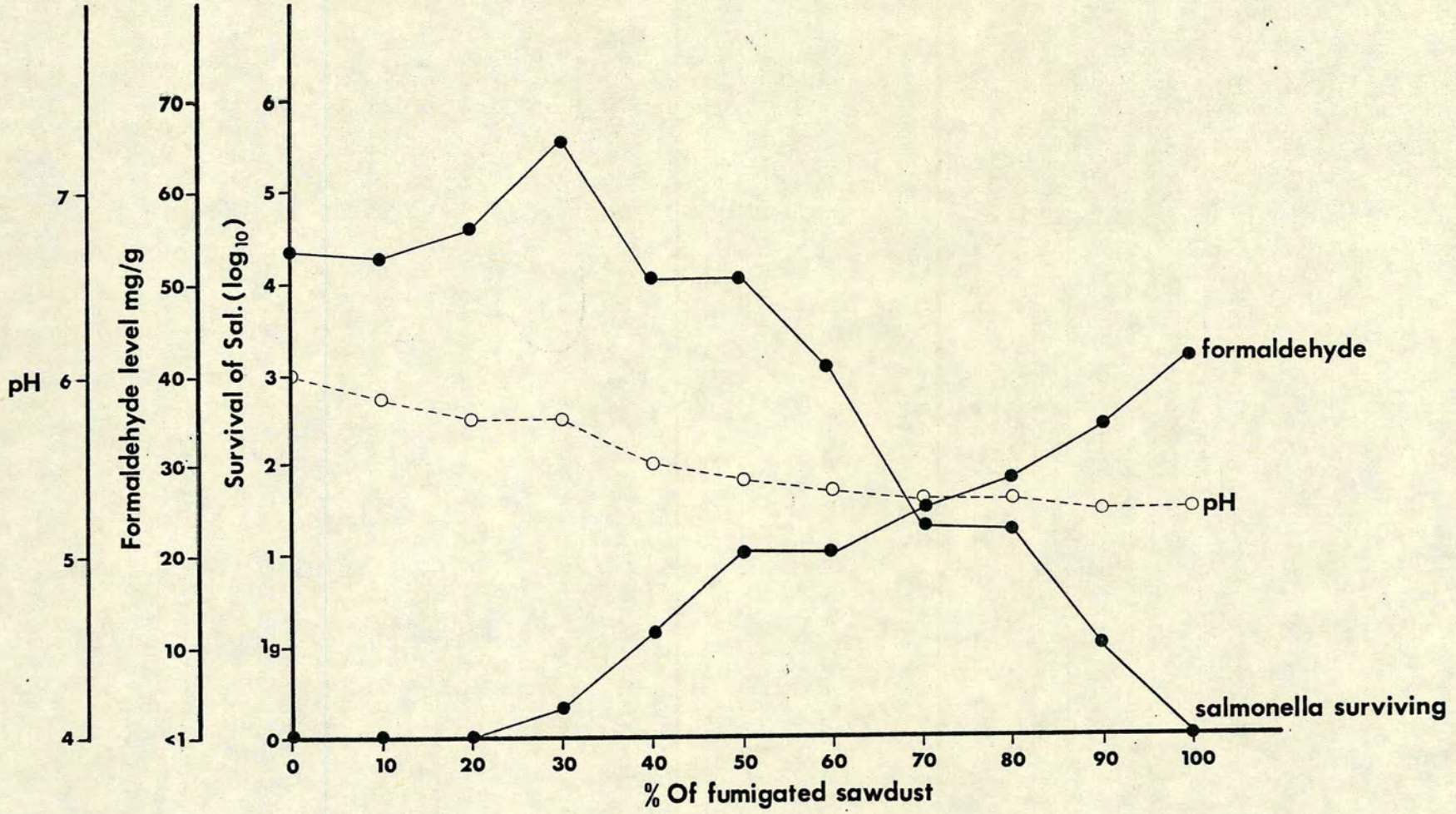
In the previous experiment the wood products all had a high level of formaldehyde as the laboratory fumigation was very efficient. In commercial houses fumigation may not be as effectively carried out so the level of fumigant will vary in different parts of the house. Therefore a laboratory experiment was designed in which the effect of variation in the level of formaldehyde on the persistence of salmonellas was determined.

Experimental Design

Sawdusts of mixed woods were collected from the Dalhousie Sawmill and fumigated in the laboratory. The fumigated sawdust was mixed with untreated sawdust to provide a series of samples with levels from 0% to 100% treated to untreated sawdust in steps of 10%. The samples were held at room temperature for 5d during which they

FIG.8.1.

SURVIVAL OF SALMONELLAS AT 48 HOURS IN SAWDUST WITH VARIOUS LEVELS OF FORMALDEHYDE



they were shaken at intervals. One gram of each mixture was then taken and incubated with 0,1ml of a 10^{-3} dilution of an overnight culture of S. typhimurium, after storage at 26°C for 2d the number of salmonellas was determined by the stated MPN-3 method. The pH value and concentration of formaldehyde were determined.

Results and Discussion

Excluding the determination of the 10% level of fumigated sawdust there was a 97.4% level of agreement between the concentration of formaldehyde as determined by chemical analysis and the level of fumigated sawdust incorporated (Fig. 8.1). Similarly the inhibition of salmonellas was directly related to the level of formaldehyde (R^2 = 91.5%). The pH value was only reduced from pH 6.0 to pH 5.4 by the addition of formaldehyde.

This experiment showed that salmonellas were inhibited when the level of formaldehyde in the litter was greater than 25mg/g.

3. The Persistence of Salmonella typhimurium in Wood Products Fumigated under Commercial Conditions (Experiment 8.3)

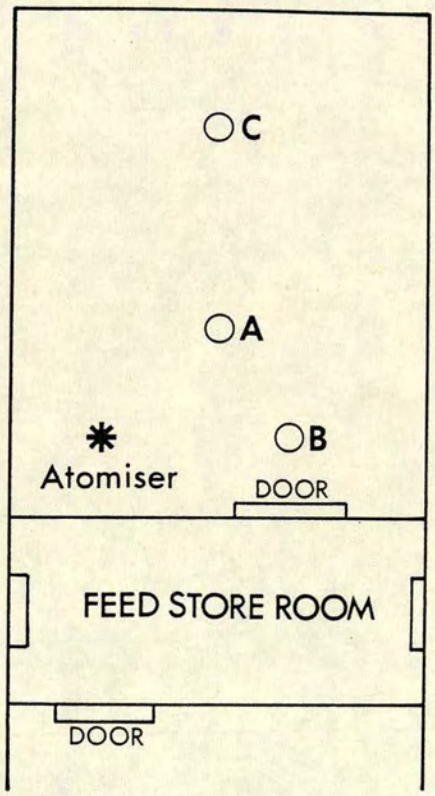
The level of fumigant in litters has not been reported in the literature therefore a trial was undertaken in a commercial house to determine the levels which could be anticipated and to ascertain the persistence of salmonellas in such materials.

Experimental Details

Samples of 1-2g of the sawdust and wood shavings of known species of trees together with a sample of the house litter were spread on individual 40m tissue culture dishes (Sterilin Ltd.). These were

FIG.8.2.

PLAN OF SITES AT WHICH WOOD PRODUCTS PLACED IN RELATION TO THE ATOMISER IN A COMMERCIAL BROILER HOUSE



in turn placed in 14cm petri-dishes and replicate sets placed at three sites of a commercial poultry house as shown in Fig. 8.2. The house was fumigated by the farm staff by atomising a solution of formaldehyde, the position of the atomiser being marked in Fig. 8.2. the effectiveness of the fumigation procedure was monitored by the method described in the General Materials and Methods (2 E*iii*).

Twenty-four hours after and on subsequent alternative days a set of samples were collected from each site together with a sample of the house litter at a distance of 0.5m from the sampling station. Sampling of the litter was repeated 2d and 4d after the chicks were placed.

At the laboratory 1g quantities of each sawdust were inoculated with 0.1ml of a 10^{-4} dilution of an overnight culture of S. typhimurium. After incubation at 26°C for 2d the numbers of salmonellas were estimated by the stated MPN-3 method.

Results and Discussion

The results presented in Table 8.2 show the inadequacy of using an atomiser to evenly distribute formaldehyde within the whole house and also shows that formaldehyde spread as a mist does not penetrate layer of 1cm of sawdust confirming the lack of penetration of litter reported by Tucker et al. (1975) and dust by Harry & Hemsley (1964).

Table 8.2

The Survival of Bacillus subtitis var globigii exposed at sites in Broiler Houses at Fumigation

Method of Exposure	No. B. subtitis recovered at site A	B	C
Exposed on Aluminium Foil	>1.00	3.60	4.04
1 drop on Petri dish	>1.00	3.34	3.90
1 drop on Petri dish covered by litter	2.00	3.90	4.45

[Control (not fumigated) = 7.00]

During the following days the level of fumigant in the litter of the house disappeared rapidly while the small quantities in the petri-dishes were subject to a slow rate of reduction.

The growth of salmonellas in these materials was inhibited in all the samples at Site A and all except the oak wood at Site B after 3d. In contrast at Site C salmonellas did grow at 24h in the house litter but not in the samples in the petri-dishes, but at 2d these bacteria grow in five of the nine sawdusts (Table 8.3).

Table 8.3

Summary of Levels of Formaldehyde in Sawdust and Woodshavings of known woods and the litter from the house (raw data in Table A8 (ii))

(a) Concentration of Formaldehyde (range of values in parenthesis)

		formaldehyde mg/g at (d):- Days			
Material	Site	1	3	5	7
Known woods	A	24 (14-35)	21.75 (0.1-50)	10.5 (13.4-16)	9.8 (8.4-12.4)
Known woods	B	52.5 (12-66)	43.75 (1-60)	5.22 (3.0-9.4)	NT
Known woods	C	60.25 (10.90)	15.25 (4-26)	3.55 (6.6-10.6)	2.8 (0.4-14)
House Litter	A	80	NT	8.6	6.2
	B	62	NT	5.6	0.6
	C	8	NT	0.4	0.2

Table 8.3 contd.

(b) Persistance of S. typhimurium in Sawdusts (species in wood specified are those supporting growth)

Material	Site	% Salmonellas surviving at (d) 1	3	5	7
All woods	A	0	0	0	0
Oak	B	0	48.1	91.6	lost
House Litter	B	0	18.1	22.1	NT
Oak	C	0	29.5	63.6	23.4
Sycamore	C	0	54.9	74.6	23.4
Pine	C	0	79.2	149.1	23.4
House Litter	C	0	97.3	149.1	NT

Once the chicks were placed the level of formaldehyde decreased at an equal rate throughout the whole house. The data generated in this experiment showed that the persistence of salmonellas in the litter directly related to the level of formaldehyde in the litter (R^2 = 90.3%) confirming the results in Experiment 8.2.

Table 8.4

The Persistence of S. typhimurium in House Litter after the Placement of Chicks

(a) Concentration of Formaldehyde

Days after placement	Formaldehyde (mg) at site:- A	B	C
2	1.6	0.2	0.1
4	1.2	0.1	<0.1

(b) Persistence of Salmonellas in Litter

Days after placement	% salmonellas surviving at site:- A	B	C
2	71.0	123.4	151.4
4	67.0	149.1	166.2

The results of this experiment show that the release of formaldehyde from the bulk of the house litter was very different from the isolated samples exposed in petri-dishes in that the inhibitory effect of the former was lost more rapidly than the latter.

Chicks are most vulnerable to salmonellas during the first 7d of life. During this first week the formaldehyde gradually disappears and so any salmonellas deposited once the level of formaldehyde has been reduced below 25mg/g could multiply. The decrease in ability to inhibit salmonellas could increase the possibility of infection of the chicks from the litter.

The next series of experiments will seek to elucidate the manner in which formaldehyde disappears from the litter.

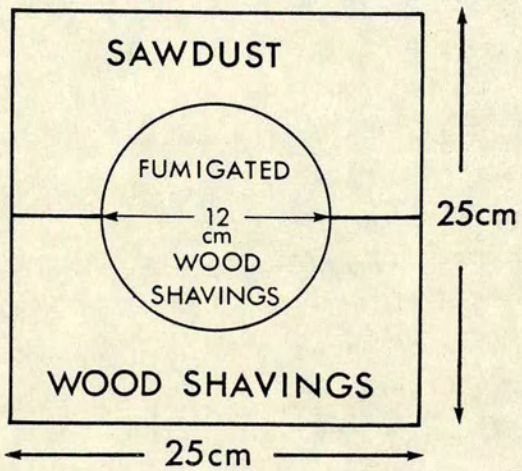
4. The Mechanism of Leaching of Formaldehyde from Poultry Litter

The previous experiment (Experiment 8.3) demonstrated that formaldehyde gradually decreased in the litters with time. The next group of experiments are designed to determine the method by which the reduction is effected.

Laboratory experiments have the advantage of providing conditions which are under the control of the experimenter, but it is important that the laboratory experiments replicate the conditions in the field. In these experiments the results of the treatments are compared in the laboratory and on the farm when applicable.

FIG.8.3

EQUIPMENT USED TO DETERMINE THE POSSIBILITY OF LATENT DIFFUSION OF FORMALDEHYDE IN SAWDUST & WOOD SHAVINGS



(a) The Reduction of Formaldehyde in Wood Products exposed in the Laboratory (Experiment 8.4)

Experimental Details

A wire cylinder was filled with wood shavings and fumigated in the laboratory and the cylinder surrounded with sawdust or wood shavings as shown in Fig. 8.3 and allowed to stand at room temperature. After 14d samples were taken from the inner and outer edges of the outside sections of the unfumigated sawdust and wood shavings and the central cylinder, at the upper and lower layers and the middle of core. A 1g quantity of each sample was weighed into a glass vial and 0.1ml of a 10^{-4} dilution of an overnight culture of S. typhimurium added, the inoculated sample being shaken and then incubated at 26°C for 2d after which the number of salmonellas was determined by the stated MPN-3 method. The level of formaldehyde and pH was also determined.

Results and Discussion

No salmonellas survived in these materials. Formaldehyde was detected in all the sawdust and wood shavings examined, the basal layer of the outer layer having higher levels than the top layer. In the central cylinder the reverse was found (Table 8.4). There was no greater variation in the levels of pH values.

Table 8.4

The Concentration of Formaldehyde and pH of Sawdusts and Wood shavings after storage to determine lateral movement

(a) Formaldehyde level (mg x 10^{-2})

Position in vertical	Position in horizontal				
	Sawdust		Central cylinder	Wood shaving	
	inner edge	outer edge		inner edge	outer edge
Top	0.5	2.5	18.75	10.0	1.75
Middle	1.25	3.0	16.25	12.5	2.0
Base	1.25	3.0	7.25	18.75	3.95

(b) pH value

Position in vertical	Position in horizontal				
	Sawdust		Central cylinder	Wood shaving	
	inner edge	outer edge		inner edge	outer edge
Top	4.9	4.9	4.6	4.75	4.6
Middle	5.2	5.1	4.75	4.6	4.55
Base	5.1	5.05	4.6	4.6	4.6

These results show that there is slight lateral movement the greater level of formaldehyde in the wood shavings suggest that this is due to volatilization as the density of the former was less than the latter. In absence of air movement it might be assumed that the formaldehyde would remain at the original site of adsorption. However it would appear that there was little loss of formaldehyde by volatalisation and a small amount due to leaching by the moisture in the basal layer.

(b) A Comparison of the Reduction of Level of Formaldehyde in the Laboratory and in a Commercial Broiler House (Experiment 8.5)

Experimental Details

Eight cylinders of chicken wire were prepared and the lower 10cm covered with plastic film. These cylinders together with eight similar without the plastic film were filled with sawdust up to a level of 20cm.

(i) Storage in Laboratory: Two of each type of cylinder were placed on a dry concrete slab and two on a damp sheet of blotting paper set on a concrete slab. These cylinders were fumigated in the laboratory and placed at room temperature under forced ventilation. The damp bases were maintained by placing a wick of muslin between the blotting paper and a beaker containing distilled water.

(ii) Exposure on a Commercial Farm: A replicate set of cylinders were transported to a commercial broiler house and placed in a group on the concrete floor and sawdust packed against the cylinder. The area was protected from accidental damage by a circle of corrugated paper . . These cylinders were fumigated in the broiler house by the method normally used by the farm staff.(2Ebi)

At 1d, 2d, 3d, 5d, 7d and 9d one of each type of cylinder was collected from the farm and the upper and lower layer sampled. At the same occasion comparable samples were taken of the litter of the house in an area to which the chicks did not have access.

(iii) Examination of Samples after exposure: One gram of each sample was weighed into a glass vial and 0.1ml of 10^{-4} dilution of an overnight culture of S. typhimurium and incubated at 26°C for

2d the number of salmonellas were determined by the stated MPN-3 sample.

The pH value, moisture content and level of formaldehyde were also determined.

Results and Discussion

Salmonellas did not persist in any of the inoculated samples. The level of formaldehyde remained high and the pH value varied very little.

(i) At the Laboratory: The set of cylinders that had been placed on damp muslin the lower layer appeared to be damper on damp muslin than the top layer. Determination of the moisture content of the sawdust confirmed the visual assessment.

Table 8.5

The Levels of Parameters in the Cylinder stored in the Laboratory

(a) Level of Formaldehyde ($mgx10^2/g$)

	Cylinders set on:-			
	Wet Concrete		Dry Concrete	
	Upper layer	Lower layer	Upper layer	Lower layer
Open cylinder	80	9.5	7.5	37.5
Sealed cylinder	50	1.7	27.5	32.0

(b) Moisture Content (%)

	Cylinder set on:-			
	Wet Concrete		Dry Concrete	
	Upper layer	Lower layer	Upper layer	Lower layer
Open cylinder	13.1	NT	8.6	5.9
Sealed cylinder	3.2	52.3	44.5	30.0

(c) pH value

	Cylinder set on:-			
	Wet Concrete		Dry Concrete	
	Upper layer	Lower layer	Upper layer	Lower layer
Open cylinder	5.4	5.7	4.9	5.45
Sealed cylinder	4.95	5.9	5.2	5.75

The cylinder with open sides dried out to a greater extent than the cylinder with a plastic film surrounding it.

When the results were computed statistically the moisture content correlated to the pH value in the "wet" cylinder at 91.2% level. The moisture content and pH value only correlated at 38.5% level but no relationship was found between the pH value and formaldehyde.

(ii) On the Farm: As in the laboratory there was very little variation in pH values although the sawdust on the farm had a slightly higher level than in the laboratory. The level of formaldehyde were lower in the farm as compared with the laboratory samples but did not vary greatly over the 9 days storage at the farm. The moisture content at the base was as expected higher than the upper layer. This equilibrium was achieved within 24h the sealed and open cylinders having similar values suggesting vertical rather than horizontal movement. When the litter in the house was examined it was noted that there were two distinct layers when the moisture content of a number of samples was determined the upper layer had a range of moisture content from 10 to 30% and the lower range 35 to 65%.

Table 8.6

The levels of Parameters in the Cylinders exposed on the Farm

(a) Level of Formaldehyde (mg x 10^2/g)

	Layer sampled	Formaldehyde at (d) 1	2	3	5	7	9
Open cylinder	Upper	2.25	2.0	0.5	0.75	1.25	1.75
	Lower	1.5	1.5	1.0	1.5	2.0	1.2
Sealed cylinder	Upper	1.25	1.75	0.5	NA	2.0	2.0
	Lower	1.25	1.50	0.75	1.25	3.0	3.1

(b) Moisture Content (%)

	Layer sampled	Moisture Content at (d) 1	2	3	5	7	9
Open cylinder	Upper	18.1	17.6	12.8	13.5	NT	20.8
	Lower	56.5	52	59.5	54.1	NT	65.4
Sealed cylinder	Upper	18.2	17.1	19.5	12.2	NT	14.4
	Lower	50.7	49.1	47.6	54.9	NT	36.8

(c) pH value

	Layer sampled	pH value at (d) 1	2	3	5	7	9
Open cylinder	Upper	5.6	5.4	5.7	5.65	5.5	5.5
	Lower	6.0	5.9	6.6	6.20	6.35	6.4
Sealed cylinder	Upper	5.6	5.4	5.5	6.0	6.4	5.75
	Lower	5.6	5.6	5.9	5.3	5.65	5.55

(d) Persistence of Salmonellas

	Layer sampled	% survivors at (d) 1	2	3	5	7	9
Open cylinder	Upper	39	20	20.0	32.6	23.4	27.2
	Lower	39	0	57.6	92.8	67.6	66.4
Sealed cylinder	Upper	0	0	107.6	32.0	32.6	23.4
	Lower	0	93.2	107.6	69.2	113.2	47.6

After the third day some salmonellas were able to persist although there was some degree of inhibiton. There was however less inhibition in the lower layer than the upper layer. This inhibition would appear to be due to the higher moisture level (R^2 = 21.1%) rather than the formaldehyde level (R^2 = 0.0%) and pH (R^2 = 3.8%) but these correlations were not statistically significant. (Table 8.6)

5. The Inactivation of Formaldehyde by Ammonia

Ammonia is know to react with formaldehyde however no information was found in the literature to indicate the effectiveness of this reaction in solution as in litter.

(b) The Titration of Ammonia and Formaldehyde in aqueous solution (Experiment 8.6)

Experimental Detail

Dilutions of a solution of ammonia (35% stated strength) and formaldehyde solution (37-41% stated strength) were prepared in glass distilled water. The solutions were mixed in the proportions shown in Table 8.7 and allowed to stand for 4h at room temperature when the level of formaldehyde was determined.

Results

Ammonia is effective in neutralizing formaldehyde in aqueous solution. (Table 8.7)

Table 8.7.

The Neutralisation of Formaldehyde by Ammonia in Aqueous Solution

Formaldehyde	Concentration of Formaldehyde (mg/ml) after addition of ammonia at:- g/ml 0.34	0.34	0.034	0.0034	0.00034
4	0.5	0.5	0.5	0.5	180
0.4	0.5	0.5	0.5	0.5	180
0.04	0.5	0.5	0.5	0.5	130
0.004	0.5	0.5	0.5	1.0	150
0.0004	0.5	0.5	0.5	8.5	320

(a) The Titration of Ammonia and Formaldehyde in Sawdust

(Experiment 8.7)

Experimental Details

Sawdust was fumigated in the laboratory, 1g quantities were weighed in glass vials and ammonia solution added as shown in Table 8.8. After thorough mixing the treated sawdusts were stored at 26°C for 24h when the level of formaldehyde was determined.

Results

Ammonia was able to neutralise formaldehyde in sawdust but the reaction was not as efficient as in aqueous solution (Table 8.8).

Table 8.8

The Neutralisation of Formaldehyde in Sawdust by Ammonia Solution

Volume of Ammonia Solution (ml/g)	Formaldehyde mg/g
3.4	4.5
0.34	5.8
0.034	>10

6. The Degradation of Formaldehyde in Sawdust by Chick Faeces (Experiment 8.8)

Chick faeces will contain ammonical compounds, this experiment will assess the effect of chick faeces on the level of formaldehyde in fumigated sawdust.

Experimental Details

A small quantity of sawdust was fumigated in the laboratory and mixed with faeces from day old chicks at various concentrations of sawdust to faeces on a weight basis. The samples were stored for 5d when the concentration of formaldehyde was determined.

Results and Discussion

Formaldehyde in the mixture of sawdust and faeces was inactivated by the added faeces (R^2 = 94.6%) (Table 8.9).

Table 8.9

The Degradation of Formaldehyde by Chick Faeces in the Laboratory

% faeces in sawdust (w/w)	Formaldehyde after 2d (mg/g)
20	8.0
30	7.0
40	6.3
50	5.7
60	3.2
70	3.6
80	1.5

Initially the quantity of faeces in the litter would be so small that it is unlikely that the reaction of ammonia and formaldehyde is a significant method of removing formaldehyde in practice. In this experiment the faeces used were of day old chicks which probably has little bacterial activity therefore the inactivation of the formaldehyde was probably due to neutralisation by ammonical compounds and to other unknown reactions with other organic compounds present.

7. The Degradation of Formaldehyde by Microbes

Bacteria which possess the enzyme formaldyhydrogenase are frequently able to metabolise methane and methanol. Therefore this ability can be exploited by using the enrichment technique devised to isolate methanologens. The ability to eliminate formaldehyde in solution was then assessed.

Experimental Details

Pelleted feed and litter (L 38) were collected from a broiler house containing chicks of 8d old. Fifty ml quantities of NMS broth in 250ml flasks were taken and the following added to two of these flasks.

1. 1g feed
2. 1g litter
3. 1g feed + 1ml of 10% sterile faecal extract
4. 1g litter + 1ml of 10% sterile litter extract
5. 1g feed + 1ml of 10% sterile faecal extract
6. 1g litter + 1ml of 10% sterile litter extract

Table 8.11

The Degradation of Formaldehyde by Bacteria derived from Poultry Litter or Feed

Period of incubation (d)	Enrichment Broth	Formaldehyde (mg/ml) after addition of Feed	Litter	Control
1	Methane alone	8.0	1.3	-
	Methane + litter extract	8.0	0.9	9.9
	Methane + faecal extract	1.3	1.2	4.5
	Methanol alone	2.0	4.2	-
	Methanol + litter extract	6.4	6.4	9.8
	Methanol + faecal extract	>1.0	9.9	9.5
4	Methane alone	4.5	0.9	-
	Methane + litter extract	4.3	0.9	NT
	Methane + faecal extract	<0.8	1.0	NT
	Methanol alone	1.0	3.0	-
	Methanol + litter extract	4.2	4.5	NT
	Methanol + faecal extract	>1.0	0.8	NT

7. 1ml of 10% sterile faecal extract

8. 1ml of 10% sterile litter extract

Methanol was added to one flask at the rate of 20g/l. The other flask was sealed with a rubber cap and flushed with methane gas which was introduced from a rubber bladder through a hypodermic syringe (Wittenbury et al., 1970).

All flasks were incubated for 5d in a shaking incubator at 26°C.

After incubation 10ml of each broth was removed to a 25ml glass vial and 1ml of a 0.1% formaldehyde added. These solutions were further incubated for 2d at 26°C after which the concentration of formaldehyde was estimated.

Results and Discussion

The enrichment broths contained bacteria able to degregate formaldehyde. The control solutions showed that sterile faecal extract also degraded formaldehyde, however bacteria cultures are able to achieve this reaction more effectively.

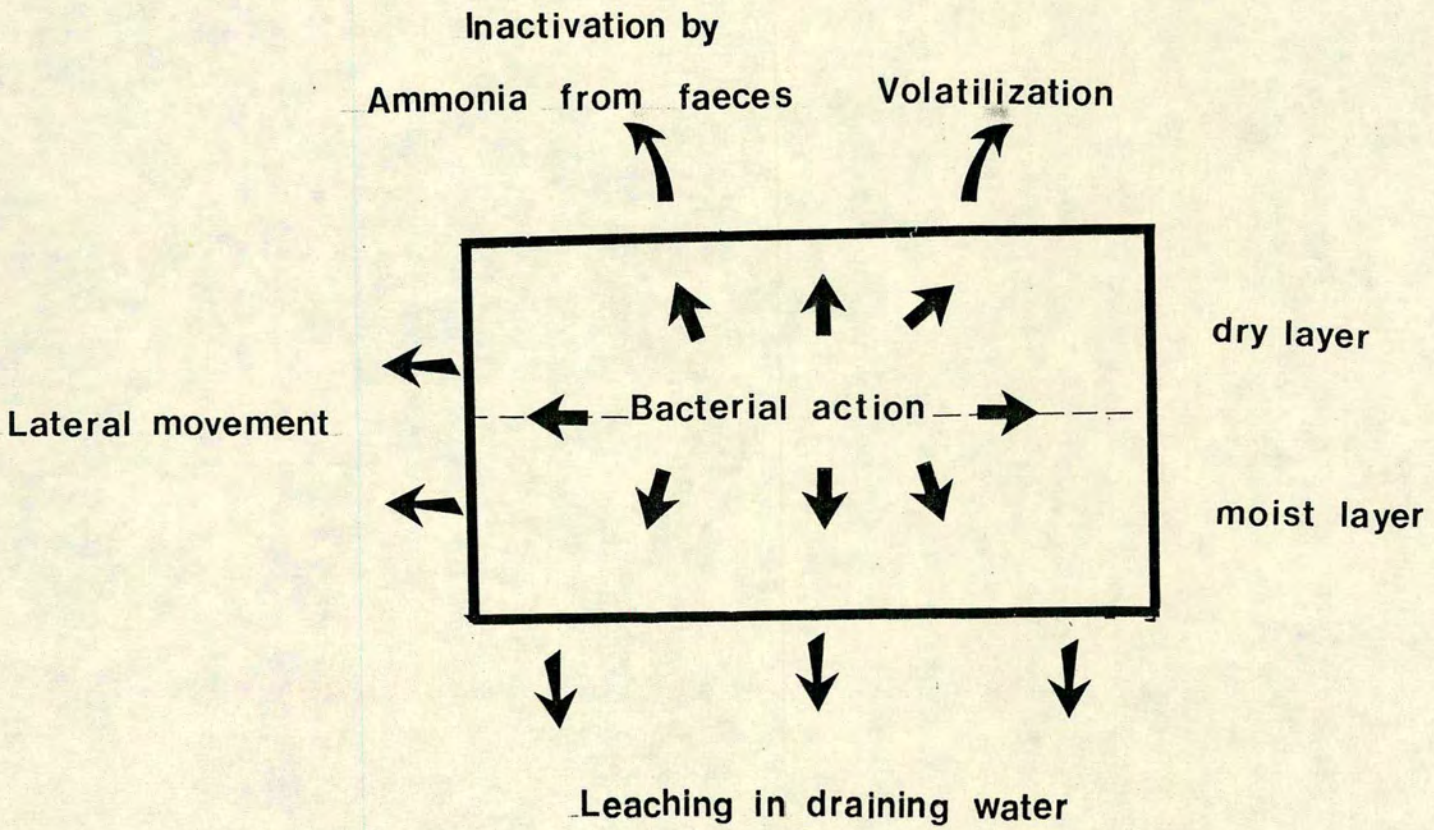
These results show that bacteria are able to degrade formaldehyde are present in the litter and feedstuff. No attempt was made to enumerate or identify these bacteria but as the method used was not necessarily specific for methanogens it cannot be assumed that only this group of bacteria are involved. The presence of organic matter will also contribute to the elimination of formaldehyde but comparison of the control solutions and the bacterially active solutions shows that the decrease in concentration of formaldehyde is greater than would be explained by the additional organic matter resulting from the presence of bacteria.

D GENERAL DISCUSSION

It has been shown that wood products are able to absorb formaldehyde which then acts as a bacteriocide, hence salmonellas deposited on the FUMIGATED litter by an infected chick will not proliferate. Wood products vary in their ability to lose this fumigant which probably explains the confusing results of Survey 1 which showed a great diversity of inhibition of salmonellas by litter in the first seven days. As the fumigant often is not evenly dispersed throughout the house (Scarlett & Mathewson, 1973) fumigation does not render the litter uniformly inhibitory (Experiment 8.3). Therefore the residual fumigant can not be relied upon to provide an inhibitory medium for the chicks for the first 2-7 days after placement, the time at which they are vulnerable to salmonella infection.

It was not possible to replicate the conditions on the farm in the laboratory as the conditions within the houses are very variable and impossible to control therefore it is difficult to investigate the rate of loss of fumigant from the litter under commercial conditions. Experiments on the farm (Experiment 8.3) showed that the woods vary in the rate at which they lose fumigant by volatilisation, oak, sycamore, and pine lose this formaldehyde before the other wood species tested. In practice wood residues of oak and sycamore are rarely included in poultry litter while pine wood is a frequent constituent. When wood shavings and sawdust are received at the farm it is of mixed woods and as each wood varies in its ability to retain formaldehyde it is not unexpected that the results

FIG.8.4 DIAGRAMATIC REPRESENTATION OF THE REDUCTION OF FORMALDEHYDE IN LITTER.



of tests undertaken on litters from farms will give inconsistent results.

In every case the bulk of litter from the broiler house behaved in a different manner to that of the isolated trial samples. Laboratory assay has shown that formaldehyde is easily removed from wood products by preparing an aqueous extract (Chapter 2, D(i)); thus it is possible that a great deal of formaldehyde is lost in water seeping from the litter. A little formaldehyde may also be lost by lateral transfer within the litter.

Bacterial action, inhibition by organic matter and neutralisation by ammonia may be other modes by which formaldehyde is lost from the litter. No methanogens have been reported from chicks faeces or poultry litter however the use of enrichment broths advantageous to these bacteria produced mixed cultures of bacteria able to degrade formaldehyde. However as the numbers of these bacteria are likely to be small in the litter it is improbable that they play a major part in the loss of formaldehyde from the litter. Similarly the amount of ammonia produced by the faeces deposited is likely to be minimal.

To date it has not been possible to quantify the loss of formaldehyde by the various routes in the litter but as shown diagramatically in Fig. 8.4 this loss can be attributed to a variety of routes of which volatilisation and leaching are probably the most important methods.

The rapid dispersion of formaldehyde from the litter precludes the reliance on the fumigant to control this source of infection for young chicks. Also the possibility of formaldehyde contributing to

the inhibitory activity of mature litter is discounted. In addition it is unlikely that formaldehyde significantly affects the bacterial mixture which ensure that the litter matures to acquire the typical inhibitory nature. Therefore if a method of artificially seeding litter to hasten the maturation process was contemplated it would appear that the residual fumigant would not interfere with the establishment of a beneficial microflora.

SUMMARY

1. Wood residues vary in their ability to adsorb and release formaldehyde.

2. The degree of inhibition of salmonellas by formaldehyde is directly related to the concentration of formaldehyde in the wood residues.

3. Formaldehyde is lost from the wood product by volatilization, lateral movement, leaching in the aqueous phase, inactivated by ammonia and degraded by bacteria.

4. It was not possible to apportion the loss to each mode of transfer.

5. It was not possible to replicate in the laboratory the conditions pertaining on the farm.

6. The rate of loss varied greatly from one poultry house to another.

CHAPTER IX

THE EFFECT OF NON-BIOLOGICAL FACTORS ON THE PERSISTENCE OF SALMONELLAS IN POULTRY LITTER

Table 9.1

Range of Concentrations of Chemicals in Poultry Litter as cited in the Literature (data in Table A9(i))

Compound	Range of Values (% DM)
Crude Protein	17.0-40.0
True Protein	23-78
N-free extract	13.7-46.0
Crude fat	1.0-5.59
Ash	11.83-18.4
Water soluble carbohydrate	2.6-3.6
Crude fibre	7.5-40.0
Total phosphate	1.1-2.39
Nitrogenous compounds	
Total nitrogen	4.04-6.8
Uric nitrogen	0.2-97.8
Urea nitrogen	0.85-30.6
Ammonia	0.39-15.0

THE EFFECT OF NON-BIOLOGICAL FACTORS ON THE PERSISTENCE OF SALMONELLAS IN POULTRY LITTER

A Introduction

No attempt has been made to define the physico-chemical parameters in litter which affect the persistence of salmonellas. Most available data has been collected in connection with the use of litter as an animal feed. Unfortunately the considerable variation in analytical techniques makes it impossible to directly compare results but they do shed light on the levels of chemicals in litter which could influence the persistence of salmonellas.

The major constituent of poultry litter is cellulose which account for 240-300mg/g determined as lignin and 400-410mg/g when measured as acidic detergent fibre carbohydrate being 46-55mg/g, crude fibre 16-27mg/g and the nitrogenous compounds 1-26.5mg/g (Appendix Table A9 (i)). The more detailed information available on the values applicable to the nutritive value of poultry litter is summarised in Table 9.1 shows a wide range of protein, fat, ash, fibre and nitrogenous compounds. The nitrogenous content includes at least twenty-two amino acids. The level of volatile fatty acids has not been reported but acetic, propionic, iso-butyric, L-butyric

iso-valeric and l-valeric acids have been detected (Watanabe, 1975). Sixteen minerals are also known to be present (Table A9 (ii) in Appendix 2). Aflatoxin was also detected by Endo et al. (1972) and Hamblin (1980) although mycotoxins have not been detected in litter in the east of Scotland even in visibly mouldy samples (Robb & Morgan-Jones, unpublished data).

The physical parameters which have already been considered at a superficial level in a previous section are moisture content and pH value (Monitoring Exercises 7.1 and 7.2) which will be seen to have been investigated by other workers. On the other hand the effect of the gaseous environment within the litter and the effect of litter temperature have been ignored. In this investigation it was decided to investigate the effect of volatile fatty acids the products of the degradation of uric acid and the additives to the feedstuffs which may accumulate in litter, as possible factors involved in the inhibitory activity of the litter.

In litter no parameter occurs in isolation and therefore the interaction of a number of these agents has been explored.

B Materials and Methods

1. Adjustment of Water Activity

(a) Solid Materials

Chambers were constructed from 4 litre polythene containers by adding two shelves of 19mm gauge galvanised wire mesh. A saturated solution of the appropriate chemical (Table 9.2) was placed in the base and a little extra solid chemical added to ensure the solution remained saturated. The material to be tested was

placed in a thin layer on 40mm tissue culture dishes. Twelve dishes could be accommodated in each container. The lid was sealed with adhesive tape and the container allowed to stand at room temperature for 2d to attain equilibrium.

Table 9.2

Chemicals used to adjust the Water Activity of Solid Materials

Chemical	Relative Humidity (appm 20°C)
Lithium chloride	11-15
Calcium chloride	31-35
Pottasium carbonate	44
Pottasium nitrate	45
Sodium chloride	76
Ammonium chloride	79
Pottasium chloride	84-86
Potassium carbonate	88
Sodium thiosulphate	95
Distilled water	100

(b) Liquid Media

The water activity of laboratory media was adjusted by the addition of glycerol or sodium chloride in the quantities listed in Table 9.2. The effect of the solutes in the media on the water activity being ignored in the calculations. Glycerol was sterilised separately while sodium chloride was sterilised in the media. The flasks containing the solutions were weighed before autoclaving and the weight loss on autoclaving adjusted with sterile distilled water.

Table 9.3

Chemicals added to Liquid Media to adjust the Water Activity

Water Activity	Grams per 1000cc	
	Glycerol	Sodium chloride
0.98	11.25	35.47
0.96	18.80	70.13
0.95	22.00	84.74
0.94	-	119.98
0.90	34.90	155.53
0.80	52.30	-
0.70	64.15	-
0.60	73.40	-

2. Determination of the Manner of "Survival" of cells in Inhibitory Solutions showing no visible growth after Incubation

Solutions known to contain inhibitory substances which showed no visible growth after the incubation period were further examined to determine whether the inoculated cells had survived in a undamaged or physiologically damaged state or had died.

The viability of the cells was determined by adding 10ml of the solution to 100ml of sterile N broth which was then incubated at 37°C for 3 days. Those solutions showing turbidity were then confirmed as containing salmonellas by streaking 1 loopful of the solution on a previously dried plate of BG agar which was then incubated at 37°C for 24h, when those colonies characteristic of salmonellas were

validated by the slide agglutination test.

The degree of physiological damage was assessed by spreading 0.1ml of the solution on a previously dried plate of BG agar and incubated for 24h at 37°C when the plates were examined and the colonies suspected to be salmonellas confirmed as above.

The results were interpretated by the definitions in Section ... summarised in Table 9.4.

Table 9.4

The survival of cells in Inhibitory Solutions showing no viable growth after incubation

Mode of Survival	Growth:- on N Broth	on BG agar
Not physiologically damaged	+	+
Physiologically damaged	+	-
Dead	-	-

C The Aqueous Phase and the Persistence of Salmonellas

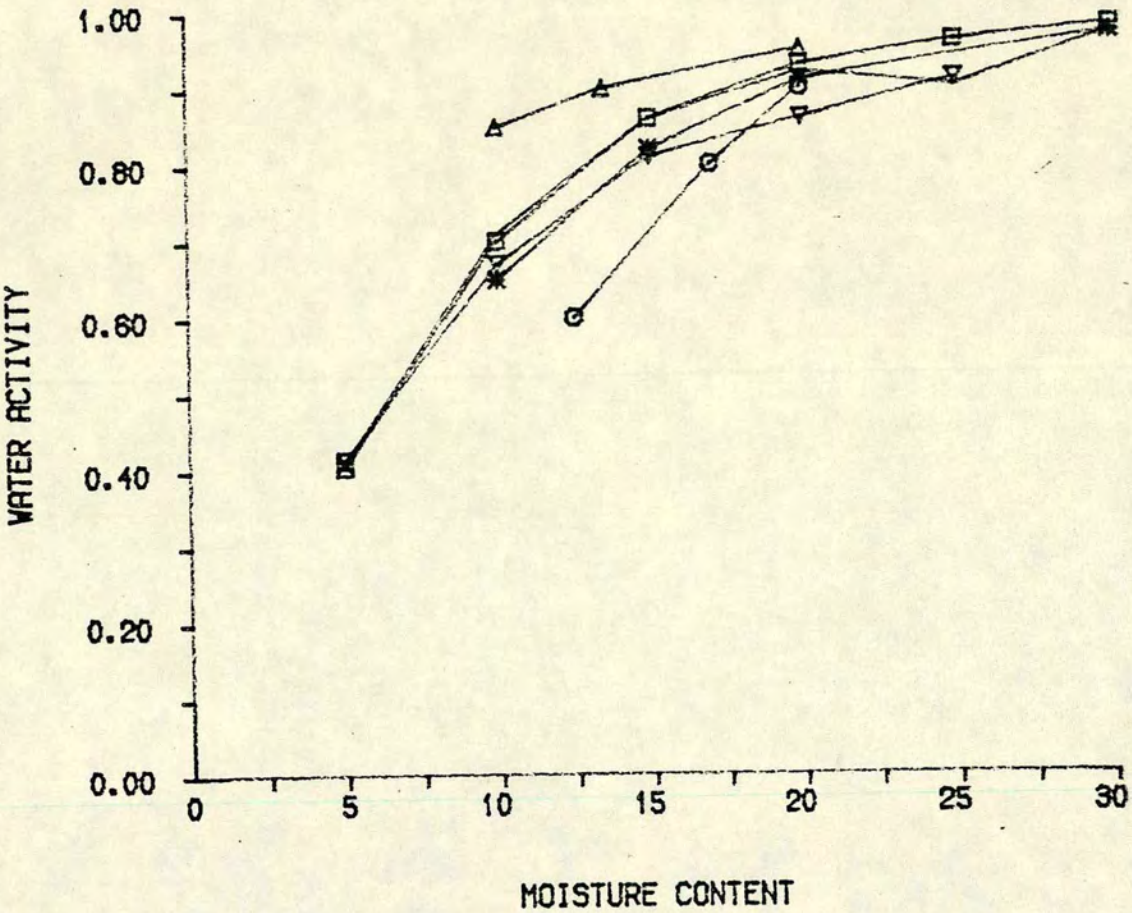
1. Review of Literature

(a) The Concept of Water Activity

As already seen the water content of litter is normally quoted in terms of moisture content or the converse-dry matter. As has been described in the General Methods (2 D 4) the moisture content is determined by heating the sample at 100°C until a constant weight is achieved and the weight loss expressed as a percentage of the original weight. This method involves the removal of water

FIG.9.1. THE RELATIONSHIP BETWEEN MOISTURE CONTENT AND WATER ACTIVITY AS CITED IN LITERATURE.

(KEY: LACEY ET AL., (1980) △ GROUNDNUT, ○ BARLEY, CARLSON & SNOEYENBOS (1970) ✱ MEAT × BONE MEAL • FISHMEAL, □ FEATHER MEAL, ▽ POULTRY MASH)



molecules loosely bound to the particles in addition to the free water between the particles and so does not give a measure of the water which is available to the microbes. However bacteria survive within the aqueous phase therefore it is more meaningful to express the water content in terms of the Equilibrium Relative Humidity (ERH%) the relative humidity at which the material neither gains or loses moisture to the atmosphere. This is in turn related to the water activity (Aw) which is defined as the ratio of water vapour of the material (p) to that of pure water (p_o) at the same temperature.

$$\text{Hence } Aw = \frac{p}{p_o} = \frac{\%ERH}{100}$$

this can be also expressed in molecular terms as

$$Aw = \frac{N_2}{N_1 + N_2}$$

where N_1 and N_2 are the number of moles of solute and solvent respectively (Labuza et al., 1976).

Water activity is the term generally accepted for this concept and so will be used in this account.

There is no published data for the inter-relationship of moisture content and water activity in poultry litter but when the moisture content and water activity of animal feeds was investigated it had been shown that the relationship was that of a paraboloid curve (Fig. 9.1).

(b) Methods of Adjusting Water Activity of Materials

The wide range of methods described for the determination of water activity in materials has been thoroughly reviewed by Troller

& Christian (1978). From their account it would appear that for solid materials the most convenient method is to exploit the property of certain chemical compounds to maintain a constant relative humidity at their interface with air. Sulphuric acid was the first chemical used for this purpose (Greenwalt, 1925; Wilson, 1921) followed by bases such as potassium hydroxide (Buston & Mellanby, 1935) and sodium hydroxide (Stokes & Robsinson, 1949). A number of saturated salt solutions have also been found to have this attribute and are more convenient to handle, also the strength of the solution cannot be modified by moisture in the environment of the test material. A wide range of these chemicals is given in tables published by Wearst et al. (1978). To determine the water activity of a material, samples of known weight are exposed to atmospheres of a range of selected relative humidities. The samples were then re-weighed and the uptake or loss of water recorded and the water activity extrapolated graphically.

The water activity of solutions can be adjusted by the addition of compounds such as sodium chloride, sucrose, glucose and glycerol (Robinson & Stokes, 1959; Taylor & Rowlinson, 1955; Grover, 1947; Grover & Nicol, 1960). The choice of solute to be added being dependent on the ingredients of the basal media and the conditions of the experiment as there can be adverse reactions with some formulations. The addition of these chemicals reduces the freezing point of the solutions which provides a convenient method of determining the water activity as these two factors are related by the following equation:-

$$- \ln Aw = 0.6934 \times 10^{-3} \times Op + 4.671 \times 10^{-6} \times \theta_F^2$$

where θ_F = lowering of freezing point (Freezing point of water - freezing point of solution) (Fero-Fontan & Cherife, 1981)

(c) The Effect of Water Activity on the Survival of Salmonellas in Solid Materials

It is essential that in solid materials that there is a small amount of free water to enable bacteria to survive. The minimum levels of water activity for salmonellas being quoted from Aw = 0.05 to Aw = 0.25 (Watts, 1945; Scott, 1978; Davis & Bateman, 1960; Christian & Stewart, 1973) but the tolerance of the bacteria to these low water activity is decreased by the presence of oxygen (Christian & Stewart, 1973). When the presence of bacteria in dried milk stored over a range of humidities was followed by Higginbottom (1962) she found that growth took place at 90-100% relative humidity but not at 0% humidity but she found that at 5-15% relative humidity bacteria survived better than at the higher or lower level of humidity. Very similar results are quoted by McDonoughty & Hargrove (1968) who found an optimal survival level was 4-7%. However for these results to be meaningful in practical terms the moisture content must be related to the water activity. In animal feedstuffs Carlson & Snoeyenbos (1970) showed that 30% moisture content represented a water activity of 0.95-0.97 a level at which they found that bacteria multiplied in contrast at 5% moisture content (Aw = 0.405-0.415) the bacterial population remained stable. In their work they found that as the moisture content level increased by 5% steps the rate of die-off decreased until 30%

was reached when the population began to increase. They also found that Salmonella enteriditis, S. heidleberg and S. tennessee all gave similar results when inoculated into feedstuffs suggesting that a change in water activity affects different serotypes in the same manner.

The temperature of storage also appears to influence the tolerance of salmonellas to water activity as Broughall et al. (1983) showed that at 12°C tolerance is at Aw = 0.95 but at 30°C survival was possible at Aw = 0.93.

In litter the salmonellas are probably on the surface of the particles and therefore it is the water activity at this interface ia crucial. Little work has been undertaken on the effect but Clayson & Blood (1957) showed that salmonellas are able to survive at Aw = 0.92 in films on glass slides.

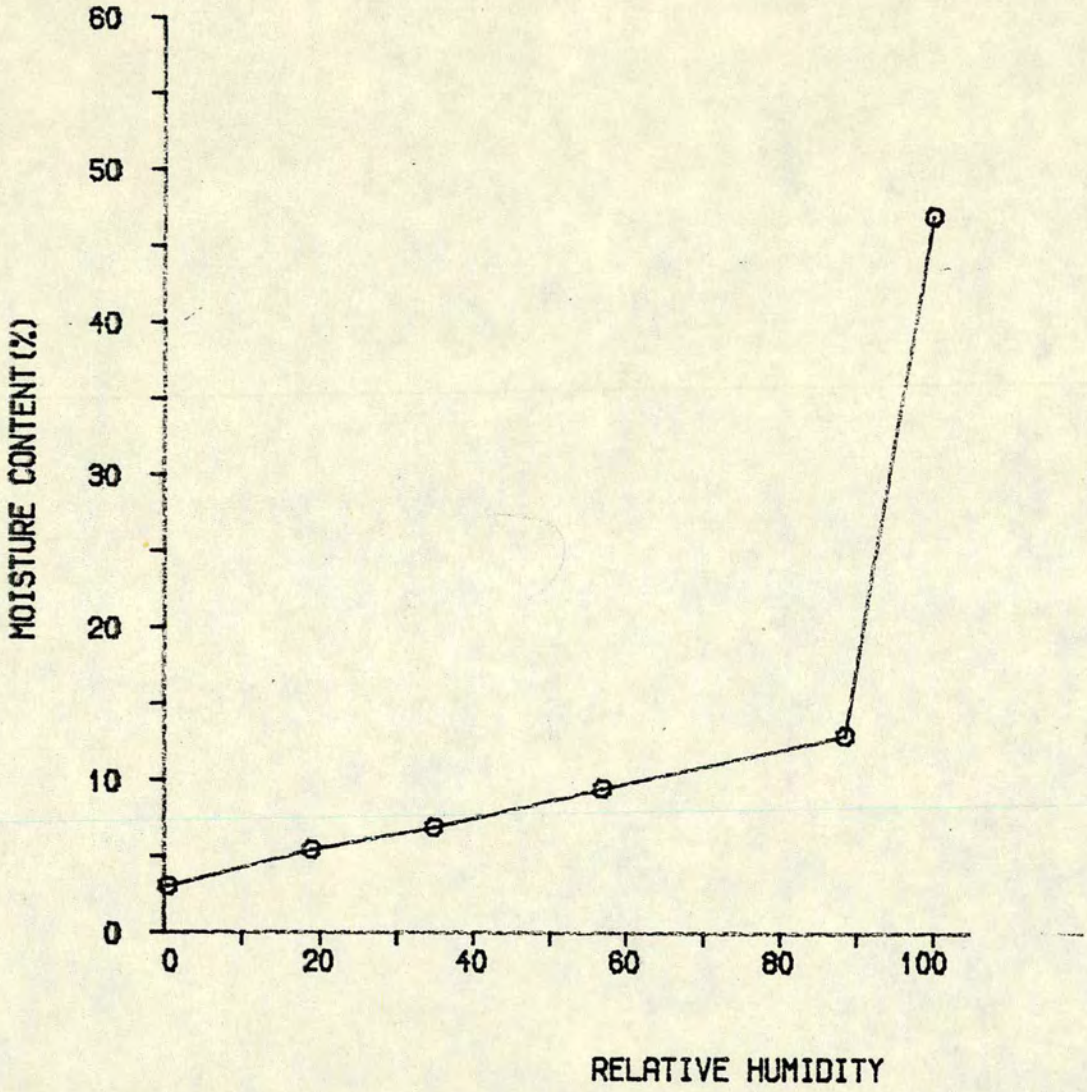
(d) The Effect of Water Activity on the Survival of Salmonellas in Liquid Media

The water activity of conventional bacterial media range from Aw = 0.9926 to Aw = 0.9992 which is within the optimal range for most bacteria (Aw = 0.98-0.995) (Chirife et al., 1982). However when adjusting laboratory media in experiments the water activity inherent in the basal media is often disregarded and in the papers published on this subject no workers specify precisely how they prepared their solutions. The variation due to laboratory preparation of media may explain some of the very slight variations in levels of tolerance reported. For example Troller & Christian (1978) gave the minimum water activity for salmonellas as Aw = 0.950 while Brown (1980) gave Aw = 0.945.

The tolerance of bacteria to water activity in liquid media is dependent on the choice of solute. When sodium chloride, potassium chloride, sucrose and glucose are used to adjust the water activity the critical level is Aw = 0.945 but when glycerol is added the level reported as Aw = 0.950 (Christian, 1955a). This variability is the result of the different modes of action of the ionic and non-ionic groups on the bacteria. Ionic solutions act by changing the permeability of the cells different compounds having slightly different activities for example glucose will allow more growth than sucrose. On the other hand non-ionic compounds such as glycerol exert their effect by binding the water in the cell so rendering the water unavailable for growth while still protecting the enzyme systems (Prior, 1978). The effect of the plasmolysis can be overcome by the action of proline although the effect of other additives to the media such as aspartic acid, asparagine, glutamic acid, glutamine and cysteine apparently increase the respiration rate and reduce the tolerance of salmonellas from Aw = 0.97 to Aw = 0.96. Inositol has also been shown to protect Serratia marcescens exposed in aerosols in atmospheres of known relative humidity (Webb, 1963) by preventing desiccation.

When considering the effect of water activity on bacteria it is essential to differentiate between the values of water activity at which bacteria will survive and those levels at which bacteria will multiply. This is particularly important when considering the data reported for the effect of water activity in liquid media but unfortunately this detail is missing in most literature on the subject.

FIG. 9.2. THE RELATIONSHIP OF RELATIVE HUMIDITY OF THE ATMOSHERE AND THE MOISTURE CONTENT OF A MATURE LITTER.



However as most researchers assess the end points by determining visible growth, it can be assumed that all the limits represented levels at which these bacteria will multiply, no attempt being made to find the levels of survival.

2. Experimental Work

(a) The Relationship of the Moisture Content and Water Activity in a Mature Poultry Litter (Experiment 9.1)

There is no data published on the relationship between the moisture content and water content in the litter. Therefore it was necesary to determine the relationship between these two parameters.

Experimental Details

Approximately 10g quantities of a mature poultry litter (L39) were suspended in muslin bags over a series of saturated solutions of known chemical for 5d. The moisture content of the samples were determined in triplicate by the Central Analytical Laboratory of the East of Scotland College of Agriculture.

Results

The relationship of the moisture content and water activity is shown graphically in Fig. 9.2. Statistical analysis of the residuals derived from the regression equation showed that the relationship is a first degree polynomial curve rather than two straight lines relationships.

(b) The Persistence of Salmonella typhimurium at a Range of Water Activity in Laboratory Media (Experiment 9.2)

Before commencing a series of experiments on the persistence of S. typhimurium under a variety of water activities had to be determined to ensure that results obtained with the selected strain in a range of laboratory media are similar to those reported for other strains in the literature.

Experimental Details

Solutions of BHI broth, BP water and N broth were prepared and the water activity adjusted with glycerol. 0.1ml of a 10^{-2} dilution of an overnight culture of S. typhimurium was added to 100ml of each solution. After incubation at 37°C for 2d the number of salmonellas was determined by the stated MPN-3 method.

Results and Discussion

Salmonellas grew in equal numbers in the media prepared according to the manufacturers instructions and the media adjusted to Aw = 0.98. At Aw = 0.96 in BHI broth and N broth and Aw = 0.95, in BP water there was a considerable drop in numbers while at Aw = 0.94 there was complete cessation of growth in all media.

Table 9.4

The Effect of Altering the Water Activity of Three Bacteriological Media on the Growth of S. typhimurium

	Salmonellas per ml in:-		
Water Activity	BH Infustion Broth	N Broth	BP Water
Control	9.04	9.04	>9.04
0.98	9.04	9.04	>9.04
0.96	6.38	7.38	9.38
0.95	5.63	6.38	6.38
0.94	<1.00	<1.00	<1.00

These results are in agreement with those reported in the literature.

As the water activity of BHI broth is Aw = 0.9932 and N broth Aw = 0.966 (Chirife et al., 1982) the slight difference in tolerance between these broths seen in Table 9.4 probably results from the variation in the water activity of the formulations of the basal media.

The mean and standard deviations of the total bacterial growth was 7.52 ± 1.78 salmonellas/ml in BHI broth, 7.96 ± 1.31 salmonellas/ml in N broth and 9.44 salmonellas/ml in BP water showing that the latter broth allowed the greater growth. Calculations of the F-ratio (1.26) showed that the media were not comparable but as N broth is the medium most frequently used in the experiments reported in the literature this broth was chosen as the undefined medium in the following experiments and M9 broth as the defined medium as no equivalent broth is quoted in the literature.

(c) The Persistence of Salmonella typhimurium in Poultry Litter and Three unrelated Materials (Experiment 9.3)

The pattern of persistence in poultry litter has been compared with three unrelated materials. Skimmilk was chosen as there is already published data on this material (Higginbottom,1962), sawdust as it is a natural product with an indigenous microflora while methyl cellulose is a manufactured product with a low bacterial load and little available nutrients.

Experimental Details

The following materials were selected for the determination of

(B) UNRELATED MATERIALS.

(KEY: △ SAWDUST, ✱ SKIM MILK, ○ METHYL CELLULOSE)

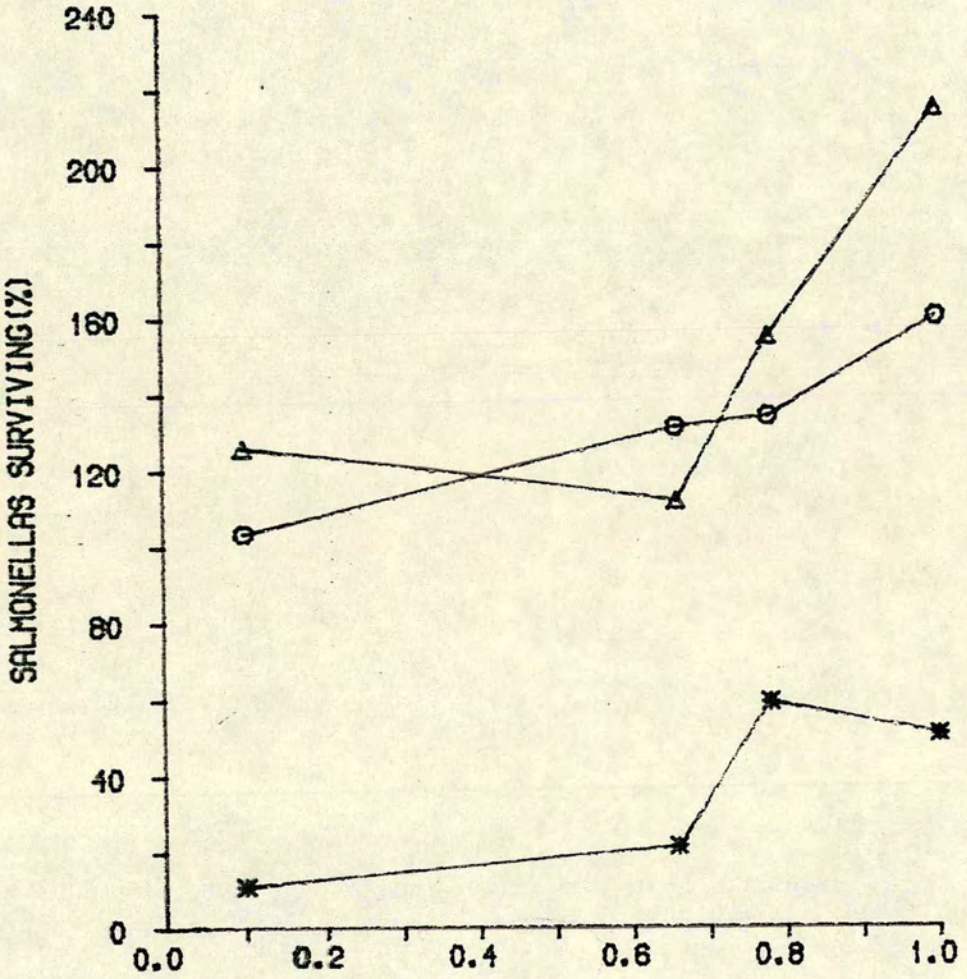
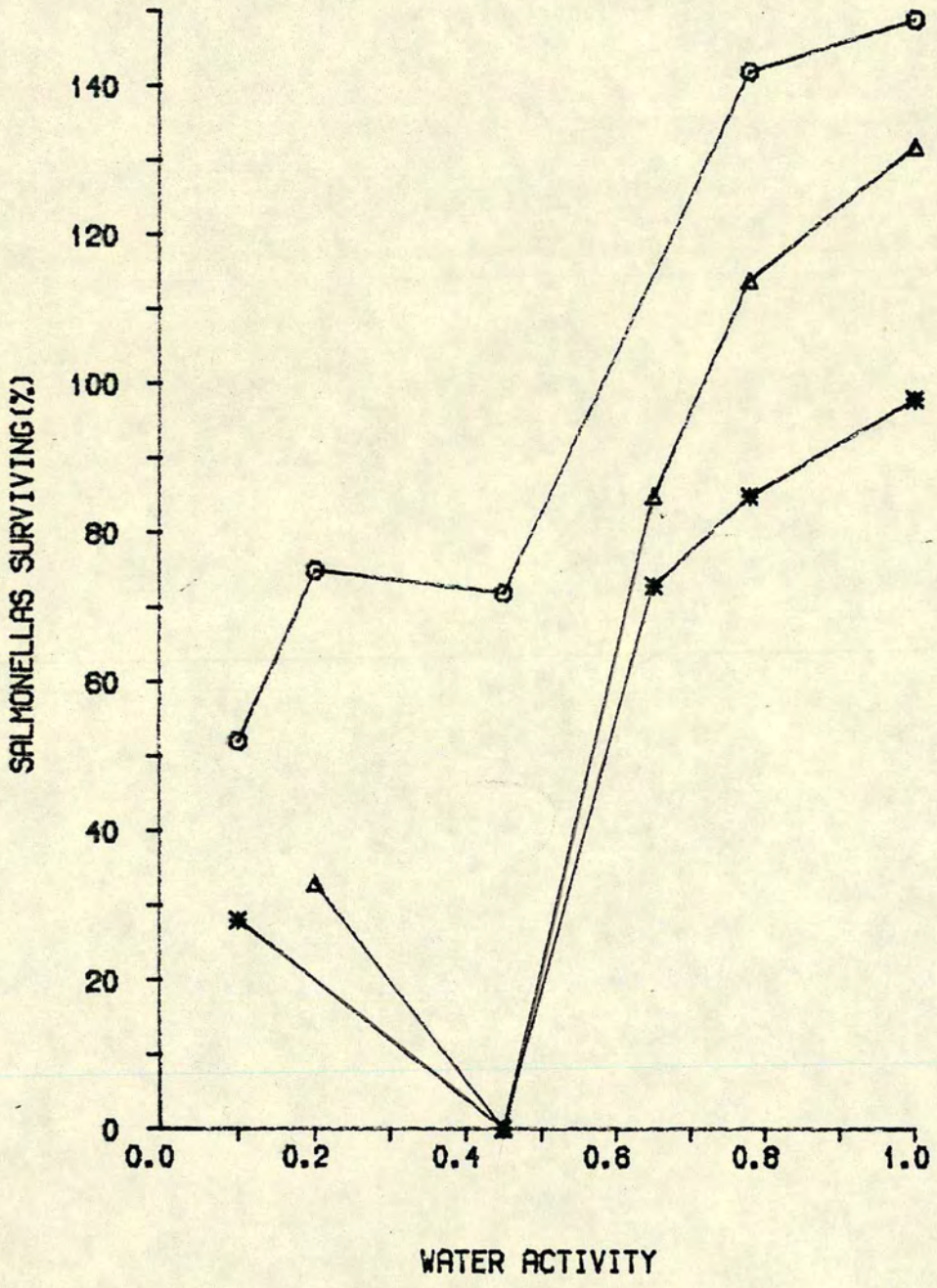


FIG. 9.3 THE PERSISTENCE OF SALMONELLAS IN POULTRY LITTER AND UNRELATED MATERIALS UNDER VARIOUS WATER ACTIVITIES.

(KEY: △ LITTER A, ✱ LITTER B, ○ LITTER B STERILE)

(A) POULTRY LITTER.



the effect of water activity on solid material were:-

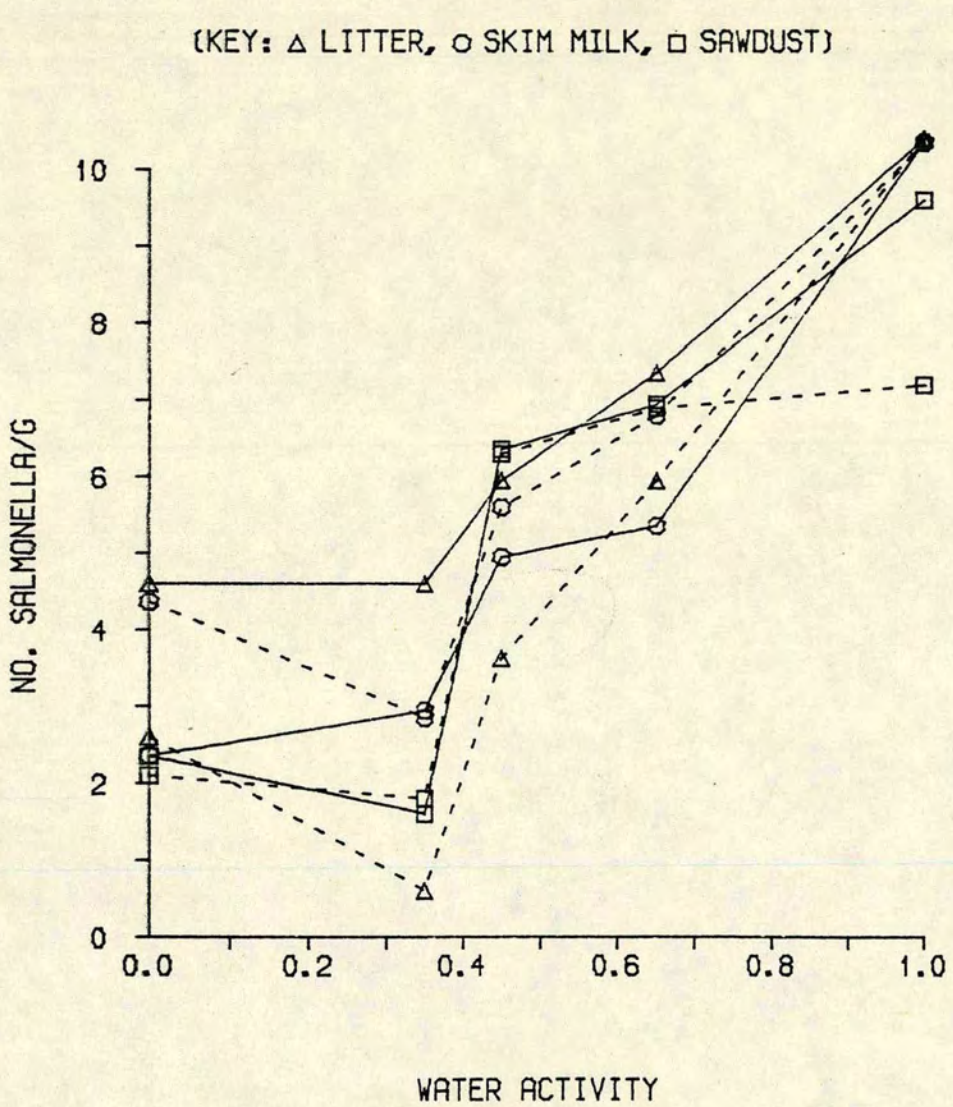
1. Poultry litter A - 15d (L30)
2. Poultry litter B - 35d (L31)
3. Poultry litter B heat treated at 121°C for 15 mins
4. Skim milk (Oxoid L31)
5. Methyl cellulose powder (BDH Chemicals Ltd.)
6. Mixed wood sawdust (Dalhousie Sawmills)

The water activity of 1g sub-samples was adjusted by placing them in chambers containing a saturated solution of chemicals. The chambers were sealed with a sheet of Parafilm (Nissin Co., Japan). After 2d storage at 26°C to allow the materials to come to equilibirium with the environment, 1ml of 10^{-4} dilution of an overnight culture of S. typhimurium was added to each sub-sample by piercing the film with a hypodermic needle (25 gauge) so ensuring that the environment of the chamber was not disturbed. The resultant holes in the film were sealed with vaseline and incubation continued at 26°C for 2d. the samples were then examined to determine the number of salmonellas using the stated MPN-3method.

Results and Discussion

As the water activity in the environment decreases from Aw = 1.00 to Aw = 0.45 in litters and Aw = 0.66 in the other materials, the numbers of salmonellas surviving also decreased. During the further decrease in water activity except for methyl cellulose the numbers of salmonellas persisting increased slightly (Fig. 9.3). The non-survival of salmonellas in the methyl cellulose is probably due to starvation accelerated by lack of available water. In litter

FIG. 9.4. THE PERSISTANCE OF SALMONELLAS IN THREE MATERIALS WITH AND WITHOUT INOSITOL



it is notable that the number of salmonellas in the sterile litter was always higher than in the litter with an indigenous microflora. suggesting that there were other factors, such as bacterial antagonism, being exerted by the litter.

(d) The effect of the addition of Inositol on the interaction of Water Activity and the persistence of Salmonella typhimurium in Poultry Litter (Experiment 9.4)

Webb (1963) showed that the addition of inositol to a culture of Serratia marcescens enabled the cells to remain viable in aerosols at intermediate humidities by preventing dessication of the cells. The following experiment was carried out to determine whether this effect could be applicable to S. typhimurium on solid material.

Experimental Details

Duplicate 1g quantities of a 22d litter (L 32) skim milk and sawdust were exposed to atmospheres with a range of relative humidities as already described in Experiment 9.3. One replicate of each pair was inoculated with an overnight culture of S. typhimurium and the other sub-sample with the culture to which inositol had been added at 5.0% (w/v). After incubation for 2d at 26°C the number of salmonellas were determined by that stated MPN-3 method.

Results

Inositol gave a small degree of protection against dessication at Aw 0.35 in the litter and sawdust but not in skim milk (Fig. 9.4). These results indicate that the decrease in persistence at Aw 0.35

may be in part due to dessication, however this data cannot be considered as convincing evidence.

3. Discussion

The pattern of growth and survival under conditions of known water activity of litter and other materials conforms to the pattern described by other workers (Higginbottom, 1962; MacDonoughty & Hargrove, 1968). The survival pattern in solid materials can be considered in three phases. At the lowest levels of water activity bacteria are able to survive but not multiply while at the middle range of water activities the salmonellas "die-out" rapidly and at the highest levels of water activity salmonellas survive and often multiply. While in liquid media it is reported that salmonellas do not grow below Aw 0.95 in solid media growth would appear to be possible at lower levels of water activity than described in broth culture. However no authors have described the effect of water activity in both types of media and none have attempted to explain these discrepancies. It is not possible to ascertain whether this is due to a variation of water activities within the structure of the solid material or whether the surface tensions exerted at the interface is less in a solid than a liquid media.

D Alkaline pH values

1. Review of Literature

The pH range which will be tolerated by salmonellas is normally quoted as pH 4.05 (Chung & Goepfert, 1970) to pH 9.6 (Brown, 1980) although at pH values of over pH 8 there is little multiplication

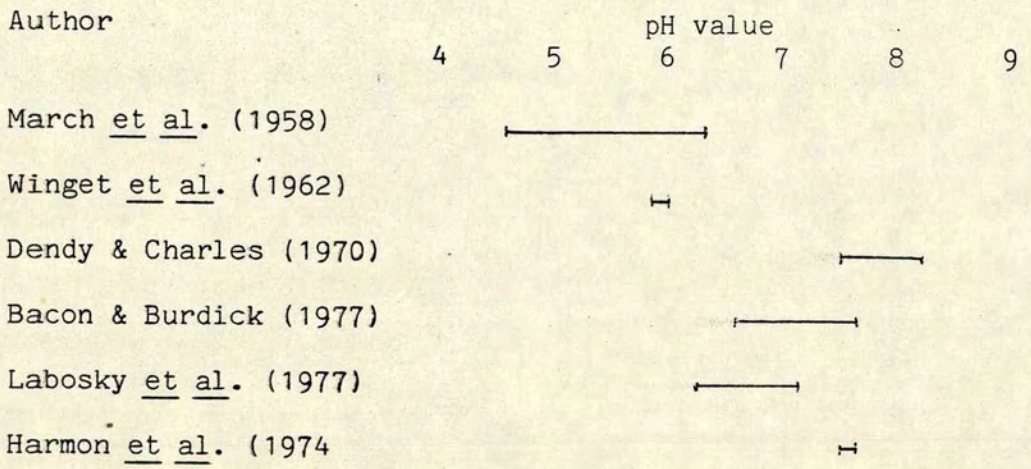
(Boxwart & Ayres, 1957). However, some serotypes grow in more restricted ranges for instance Salmonella paratyphi has a range of pH 4.5-7.8 (Derby, 1921; Brown, 1980), S. typhosa from pH 6.2-7.6 (Derby, 1921) and S. schottmeierlleri from pH 4.5-8.0 (Derby, 1921).

The range of tolerance of salmonellas at the extremities of the range of pH levels is affected by the particular chemical used to adjust the medium. Subramanian & Marth (1969) showed that skim milk adjusted over a range of pH 6.7 to pH 4.0 with citric acid was more inhibitory than when adjusted by lactic acid while hydrochloric acid was the least inhibitory of these acids. The tolerance to hydrochloric acid was confirmed by Chung & Goepfert (1970) who reported that in their hands salmonellas survived at pH 4.05 with hydrochloric acid but only at pH 5.5 when propionic acid was used to adjust the media. More recently Rubin (1978) has shown that a mixture of lactic and acetic acids was more inhibitory to S. typhimurium than the sum of the two acids separately.

The tolerance of a bacterial strain can be extended by a gravity plate method, Huhtanen (1975) reporting an increase of the pH range by 0.8-1.0 units by this method. He found that this attribute was lost quickly when the modified strain was then cultured in tryptic case-soya broth prepared to the manufacturers specifications.

In litter the growth of bacteria is probably on the surface of the particles and therefore the tolerance of the salmonellas at the surface/air interface is important. Such a situation has

Fig. 9.5 The Range of pH values in litter cited in literature



been studied by Matches & Liston (1972) who found that S. heidleberg was able to grow at pH 8.0, S. typhi , pH 9.0 for S. derby.

At the limits of pH level which salmonellas survive there appears to be an increase in the generation time, the log phase increasing from 72min for S. pullorum and 46min for S. oranienburg and S. senftenberg at pH 6 to 150min, 72min and 53min respectively at pH 8 (Boxwart & Ayres, 1957).

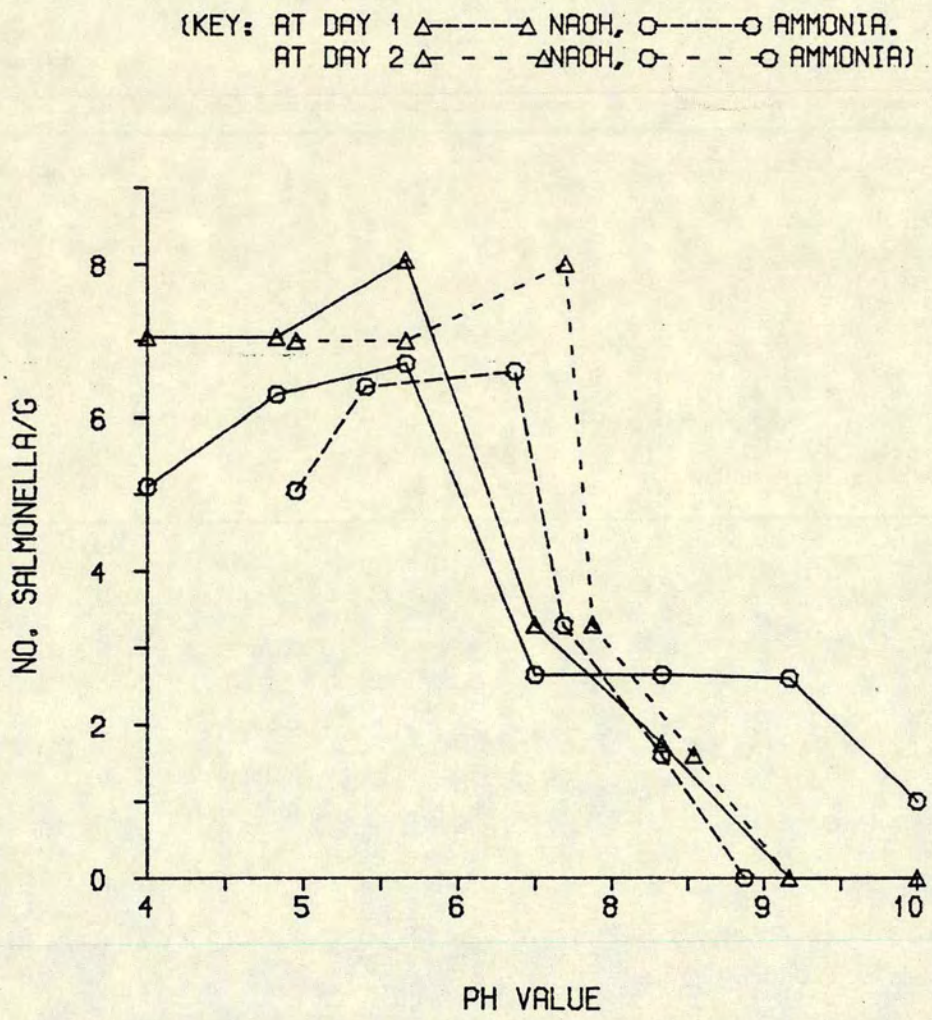
It is generally accepted that the alkalinility of the litter is responsible for the inhibitory effect of older litters. However there is a dearth of information of the pH values of litter as can be seen in Fig. 9.5 which shows that although a range of pH 5.1 to 8.6 is reported in the literature the majority of samples fall between the limits pH 7-8.6.

2. Experimental Details

(a) The Persistence of Salmonella typhimurium in Nutrient Broth with the pH level adjusted with Ammonia or Sodium Hydroxide (Experiment 9.5)

When preparing laboratory media it is customary to adjust the pH level to alkaline values by sodium hydroxide but in litter this effect appears to be produced by ammonia dissolving in the aqueous phase of the litter. The review of literature has shown that the chemical used to adjust the pH level of a medium may affect the tolerance of salmonellas to the resultant level of survival. This experiment will show if such a variation occurs in N broth when inoculated with the chosen strain of S. typhimurium.

FIG.9.6. THE GROWTH OF SALMONELLAS IN NUTRIENT BROTH PH ADJUSTED WITH SODIUM HYDROXIDE OR AMMONIA SOLUTION.



Experimental Details

Twelve 100ml quantities of N broth were autoclaved then using 2N ammonia solution or 2N sodium hydroxide (prepared in sterile water) the pH level of these solutions was adjusted to pH 7, 7.5, 8.0, 8.5, 9.0 and 10.0. Fifty ml quantities of each solution was transferred aseptically to a fresh sterile 100ml conical flask and 0.1ml of a 10^{-3} dilution of a overnight culture of S. typhimurium added and the flasks incubated at 26°C for 2d. After incubation the number of salmonellas was determined by the stated MPN-3 method.

Results and Discussion

The results showed that there is no difference in the pattern of growth of salmonellas in the alkaline media irrespective of the chemical used to adjust the medium (Fig. 9.6).

It can also be seen that over pH 7.5 there was no multiplication of the salmonellas but these bacteria were able to survive up to pH 8.5-9.0.

E The Gaseous Environment

1. Review of Literature

Litter is a situation of active metabolism so it may be anticipated that the micro-environment contains many sites with a low oxygen tension. Salmonella as facultative anaerobes (Cowan, 1974) would be favoured in such situations where they would compete well with obligate aerobic bacteria.

In 1935, Burrows & Jordan, despite the difficulties in

in measuring E_H changes, showed that bacteria were tolerant to changes in the oxygen tension. Burrows (1941) went on to show the association between a decreasing oxidation reduction potential with a corresponding decrease in the growth of aerobic bacteria. The importance of growth in a slightly reduced E_H to Salmonella typhimurium has since been demonstrated by Oblinger & Kraft (1973) who showed that in a non-aerated system the initial rate of growth of salmonellas was more rapid than in a corresponding aerated system. In these experiments the maximum growth in the former took place within 80h while in the latter 120h was required. When S. typhimurium was grown in broth in competition to Pseudomonas fluorescens the growth pattern of the salmonellas in an aerated system was more akin to the growth of the pure culture in an aerobic system suggesting that the stimulatory effect of the pseudomonads operated by reducing the oxygen tension of the media. Similarly in solid materials Lamprecht & Elliott (1974) showed that in an environment of pure oxygen the survival time for salmonellas in fishmeal was reduced. They also showed that moisture content and temperature affected the length of time for which salmonellas could survive in oxygen in this material. They found that at moisture contents of 8% and 12% survival times were greater than 5% and 30% the shortest time being at 20%. In air the salmonellas were able to survive for a greater period of time than in pure oxygen. However, when grown in pure carbon dioxide the growth of S. typhimurium is reduced to 85% of the growth in air (Eklund & Jarmund, 1980).

A great deal of the investigational work on the effect of gaseous environment on pathogens has concentrated on the storage of foods in a wide range of packaging material which are not comparable to the situation in litter. However the paper of Shaw & Nichol (1969) although considering the survival of salmonellas in food gives useful information which is applicable to litter. These workers sprayed S. organienburg at the rate of 3.0 bacteria per cm^2 on meat slices which they then exposed in a variety of gaseous environments. In anaerobic conditions (100% nitrogen) the growth was half that attained in air, increasing the oxygen to 0.6% or 18.7% resulted in a reduction in growth compared to that in air of 24% and 27% respectively.

There are many technical difficulties involved in determining the effect of gaseous environments in litter as it is impossible to ensure that the conditions are uniform throughout the material which is metabolically active. Even in laboratory media investigations of the effect of oxygen tension in the environment on the growth of bacteria requires normally specialised equipment but the gel-stabilised gradient method of Wimpenny et al. (1981) is a simple inexpensive technique and was explored in this investigation.

2. Experimental Work

(a) The Growth of Salmonella typhimurium in Gel-stabilised Gradient System (Experiment 9.6)

Gel-stabilised gradient systems enable the growth of bacteria over a limited range of oxygen tension when the method as described

by Wimpenny et al. (1981) therefore rusty nails were added to increase the anaerobosis of the lower layers of the column.

Experimental Details

Two duplicate, 1 litre straight sided beakers of 20cm height were added 800ml of the following:-

(a) N broth with 0.7% Oxoid No. 3 agar (0.7% N agar)

(b) BP water with 0.7% Oxoid No.3 agar (0.7% BPW agar)

Rusty iron nails were placed in the base of one beaker of each media.

Ten ml of an overnight culture of S. typhimurium was mixed into each beaker. After incubation of 5d at 26°C a pipette with the tapered end removed was used to extract a column of agar from the beaker. From this column 10cc quantities of agar from the base and the top of the cylinder were extracted and added to 10ml of Ringer's solution. The numbers of salmonellas were determined by the stated MPN-3 method.

Results

Assuming that the conditions quoted by Wimpenny (1981) were established in this laboratory it must be concluded that under conditions of reduced oxygen tension, there is a slight decrease in numbers when 0.7% BPW agar was the medium but not when 0.7% N agar was used. Reduction of the oxygen tension at the base of the column by the addition of rusty nails resulted in a 2 $\log_{10}$ reduction in numbers in the beaker containing 0.7% N agar but only

a slight reduction with 0.7% BPW agar. (Table 9.6)

Table 9.6

The Number of Salmonellas in Gel-stabilised gradient column

Content of gel	No. of Salmonellas/ml Top	Base	Difference (Top-Base)
N broth + 0.7% agar	10.38	10.38	0
BP water + 0.7% agar	7.97	8.97	+1.00
N broth + 0.7% agar and nails	10.38	7.56	-2.38
BP water + 0.7% agar and nails	9.38	8.88	-0.5

As the conditions in this experiment are very different from the system in litter the following experiment will investigate the effect of four gaseous environments on persistence of salmonellas in litter.

(b) The Persistence of Salmonella typhimurium in Litter exposed to Four Gaseous Environments (Experiment 9.7)

There are many factors which suggest that conditons within litters are micro-aerophylic that the persistence of S. typhimurium in litter stored under four gaseous environments was investigated. The difficulty of removing air by vacuum without disturbing the material made the use of a method based on the commercial anaerobic system manditory although it restricted the choice of gaseous environments.

It was decided to inoculate one set of litter immediately they were received from the house and then expose them to the gaseous environments while allowing the other set of samples to equilibriate

with the environment for 3d prior to inoculation. This enabled the former to assess the effect exerted by the indigenous microflora as found in the litter of the house while the latter enabled some selection of those groups of bacteria favoured by these particular environments.

Experimental Details

Samples of litter were collected from houses containing birds of 8d, 22d, 30d and 42d (L33-36). Two 1g quantities of each litter were placed on 14cm tissue culture dishes in each of four gas jars. One sample of each litter was inoculated with 0.1ml of 10^{-4} dilution of an overnight culture of S. typhimurium.

The following gaseous atmospheres were established:

1. Anaerobic conditions - BBL Gas Pak
2. 5-15% oxygen + 5-12% carbon dioxide in hydrogen - BBL Camyl Pak
3. as 2. + 100cc air (jar under slight pressure)
4. Aerobic - air, jar with valves open.

After 3d incubation at 26°C the jars were opened and the inoculated litter examined to determine the numbers of salmonellas persisting by the stated MPN-3 method. The remaining samples were inoculated as before and the gaseous environments re-established. After a further 3d incubation at 26°C the numbers of salmonellas persisting in these samples was estimated by the stated MPN-3 method.

Results

Storage prior to incubation rendered all the litter less inhibitory to salmonellas with the exception of the litter of 8d exposed to anaerobic and 5-15% oxygen conditions. It would therefore appear that these bacteria implicated in inhibition appear to "die-out"

in all four environments. Generally a low level of oxygen allowed greater numbers of salmonellas to survive. (Table 9.7)

Table 9.7

The Numbers of Salmonellas Persisting in Litters stored in Four Gaseous Environments

Age of Litter (d)	Duration of storage prior to incubation (d)	Gaseous environment			
		Anaerobic	5-15%0_2	6-16%0_2	100% Air
8	0	1.66	7.38	3.36	2.66
22		1.38	2.66	5.97	2.38
30		1.38	2.97	4.97	2.38
42		1.38	1.26	3.66	1.36
8	3	1.36	5.97	7.63	8.63
22		4.36	7.32	5.97	5.36
30		5.36	7.36	9.36	6.63
42		6.46	2.36	4.63	3.63

These results show that the gaseous environment does affect the survival of salmonellas. The situation in nature is likely to be more complex with the modified environment affecting both the multiplication of salmonellas and the genera of bacteria able to inhibit salmonellas to a greater degree than in this experiment.

F Temperature

The minimum temperature at which salmonellas can multiply has been recorded as 5°C and the maximum 55°C (Troller & Christian, 1978). However the tolerance of these bacteria to extreme conditions appears to be in part dependent on the medium in which the salmonellas

are suspended as Angelotti et al. (1961) recorded minimum temperatures for multiplication in a variety of foods from 6.7°C to 10°C similarly Matches & Liston (1968) quotes 8°C for growth in fish and 11°C in crab meat.

All workers agree that the optimal temperature for the growth of salmonellas is 37°C but in litter it has been shown that these bacteria persist better at 11°C and 25°C than at 38°C (Williams & Benson, 1978b).

As the effect of temperature will be considered in conjunction with other parameters in later experiments (Experiments 9.15, 9.16 and 9.18) it is not considered as a separate factor at this stage of the investigation.

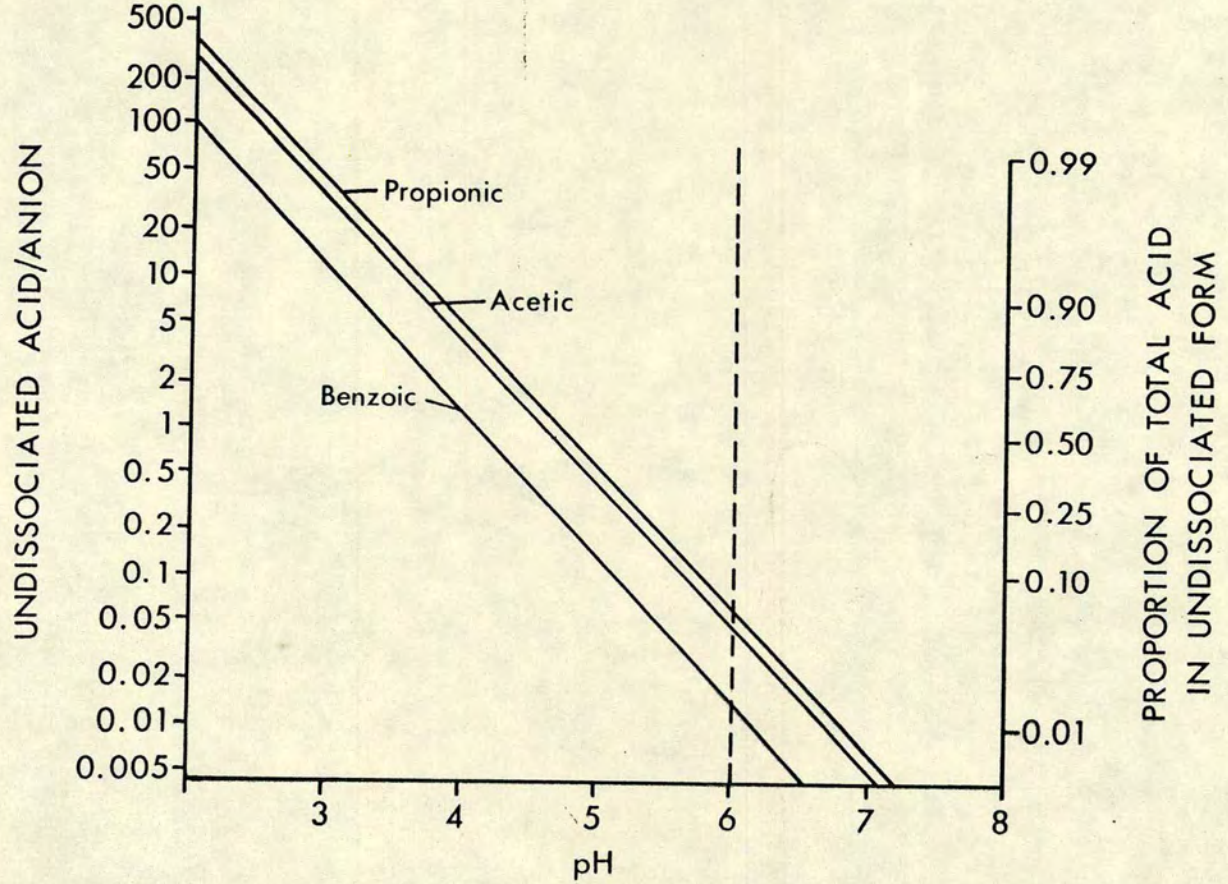
G Volatile Fatty Acids

1. Review of Literature

The inhibitory effect of volatile fatty acids (VFA's) has been recognised since the early 1960's when Meynell (1963) and Bohnhoff et al. (1964) demonstrated that in the caecum and colon of both man and animals anaerobic bacteria produce acetic, propionic and butyric acids that inhibit salmonellas. Meynell (1963) also showed that this anti-bacterial activity of VFA's to Salmonella typhimurium and S. enteritides in mice was greater in anaerobic conditions than in aerobic conditions. A similar effect was reported by Bohnohoff et al (1964) but Brownlie & Grau (1967) and Freter (1974) suggested that there is a possibility that other chemicals are also involved in the inhibitory effect of intestinal contents on salmonellas.

FIG.9.7.

The relationship between pH and proportion of undissociated acid in a solution of an organic acid. (after Troller & Christian, 1978)



Barnes et al (1979) has shown that VFA's have a major role in the anti-salmonella activity in the gut of the young chick. These workers have also shown that in VL broth, which has a low redox potential, acetic and propionic acid inhibits S. typhimurium within 24h at pH 5.8.

The inhibitory activity of VFA's is dependent on the dissociation of these acids (Simon & Beevers, 1952; Ingram et al., 1956; Goepfert & Hicks, 1969; Galbraith et al., 1971) as seen in Fig. 9.7 The dissociation is greatest at levels over pH 6.5-7.0 decreasing at acid pH levels (Troller & Christian, 1978). In addition the inhibitory action of VFA's is related to the chain length of the molecule, those with short chain length being more inhibitory, those with long chain lengths (Khan & Katamay, 1969; Goepfert & Hicks, 1969; Wolin, 1969; Fay & Fariar, 1975).

The inhibition of VFA's can be reversed in broths by the additon of bovine serum albumin and surface active agents (Willett & Morse, 1966), calcium, magnesium, cholate and ergecalcifial (Galbraith et al., 1971).

In solid materials the antimicrobial activity is similar to that in broths but generally less marked. For instance in fish-meals the level of salmonellas can be reduced slightly by the addition of formic acid (0.5%), acetic acid (0.2%) propionic acid (0.5%) and butyric acid (0.5%) (Lamprecht & Elliott, 1974) similar results being shown in meat and bone meal (Khan & Katamay, 1969).

In litter a mixture of 60% acetic acid and 40% propionic acid added to wood shavings at the rate of 3% (w/w) reduced the mould

count for 2w and at 1% and 3% retarded the total bacterial count for 1 week. The 3% level initially reduced the pH to 3.9 but this gradually rose to a neutral pH. In this trial the effect of these treatments on the survival of salmonellas was not considered (Parkhurst et al., 1974).

From this review of literature it can be seen that VFA's are inhibitory under acidic conditions but alkaline conditons prevail in litter. As there is a dearth of evidence on the action of VFA's in alkaline conditions this will be pursued in the following experiments.

2. Experimental Work

(a) The Confirmation of the Results of Barnes et al. (1979) and the Extension of this data to Alkaline Conditions (Experiment 9.8)

In 1979 Barnes et al. in their investigation on the inhibitory activity of the chick caecum determined the effect of VFA's over a range of pH values from pH 5 to pH 6. This experiment repeats their experiments and extends the observations to pH 9.0.

Experimental Detail

The method detailed by Barnes et al. (1979) was repeated using Salmonella typhimurium as the test organism, the pH value of the VL broth was adjusted to the alkaline pH value with 0.1M sodium hydroxide. The growth of salmonellas measured by the turbidity of the solution rather than by visual assessment.

Table 9.8

The Effect of Volatile Fatty Acids on the Growth of Salmonella typhimurium in Broth Culture at pH range pH 5 to pH 9 (data of Barnes (1979) in parenthesis)

VFA's	Concentration µmol/ml	Incubation time (h)	Nephalometer readings at pH:- 5	6	7	8	9
Acetic acid	15	5	20 (-)	14.7 (++)	22.3	11.2	8.0
		24	77.2 (-)	100+ (+++)	100+	100+	
	30	5	20 (-)	21 (-)	17.7	10.2	8
		24	63.5 (-)	100+ (+++)	100+	100+	5.5
Propionic	15	5	20 (-)	16 (-)	23	9	6.5
		24	74 (-)	100+ (+++)	100+	98.5	5
	30	5	21 (-)	19 (-)	15.2	12.3	8.9
		24	66.5 (-)	100+ (++)	100+	100+	5
Control	-	5	22 (++)	17 (++)	22	13	8
		24	36.5 (++)	100+ (+++)	100+	100+	5.5

Results and Discussion

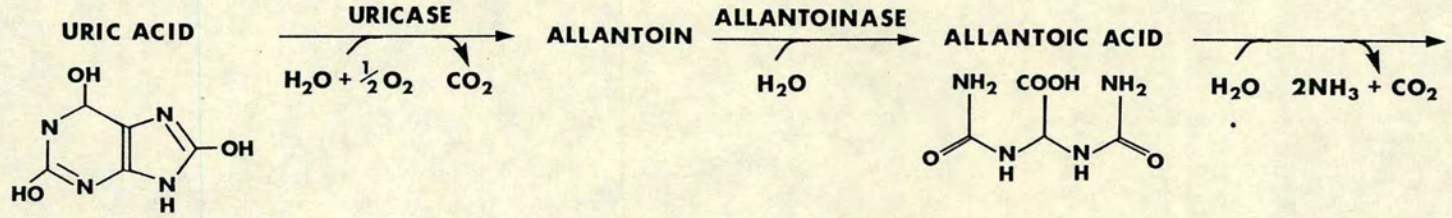
As anticipated the VFA's inhibited the growth of salmonellas at pH 5 at both 15 and 30mg/ml while at pH 6 the salmonella was only inhibited by acetic acid at 30mg/ml (TABLE 9.8). The results at pH 6 differ from the findings of Barnes et al. (1979) who found a greater degree of inhibition. However as shown in Fig. 9.7 a small difference in pH could lead to a marked difference in dissociation at this level with the resultant variation in inhibitory effect. Other VFA's (iso-butyric, iso-valeric (Shrimpton, 1963; Watanabe, 1975) n-butyric and n-valeric (Watanabe, 1975)) have all been isolated from chick faeces but as dissociation is affected by the chain length of the acid it is unlikely that these compounds will inhibit salmonellas at the alkaline pH of poultry litter.

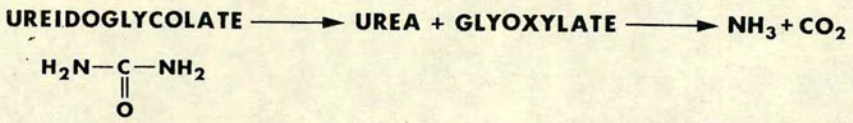
H Nitogenous Compounds and the degradation of these compounds

(1) Review of Literature

Uric acid, the main nitrogenous compound in chicken faeces is degraded aerobically or anaerobically to allantoic acid, urea, ammonia and carbon dioxide (Fig. 9.8). Schefferle (1957) and Bachrach (1957) agree that although some bacteria are able to degrade uric acid to ammonia, the reaction is probably more often in two steps, one group of bacteria degrading uric acid to urea and the second group transforming the urea to ammonia. The species able to effect the change from uric acid are Corynebacterium sp., Nocardia sp., Streptomyces sp., Alcaligenes sp., Achromobacter sp. (Schefferle, 1957), Klebsiella pneumonia, Serratia kiliensis, Bacillus sp., Pseudomonas aeruginosa, (Rauf & Lamprey, 1968) Proteus sp., some staphylococci and micrococci.

FIG.9.8. THE DEGRADATION OF URIC ACID TO AMMONIA & CARBON DIOXIDE





The further degradation to ammonia being undertaken by strains of Corynebacterium spp., Micrococci sp., Achromobacter spp., Cytophaga sp. (Schefferle, 1965), Micrococcus urea, Sarcina urea and Bacillus lentas (Mahmoud et al., 1979), Aerobacter aerogenes, Klebsiella pneumonia, Serratia kiliensis, Pseudomonas aeruginosa and Proteus sp. (Rauf & Lamprey, 1968). Mohmond et al. (1979) showed that 30°C was the optimal temperature for this reaction and also demonstrated that alkaline conditions are produced, Micrococcus urea and Sarcina urea modifying the pH from pH 5.5 to pH 9.0 and Streptomyces sp. to pH 8.5. Salmonellas are unable to effect this degradation an attribute which is routinely exploited in the urease test (Christensen, 1946) used when confirming the isolation of salmonellas.

While the greatest proportion of the ammonia produced is dissolved in the aqueous phase of the litter it is possible that some of the ammonia ions could be incorporated into amino-acids such as ketoglutarate which may be further degraded to chemicals which in turn inhibit Gram-negative bacteria (Halpern & Umburger, 1960) but little is know of this reaction.

All the products of the breakdown of uric acid have been detected in poultry litter, the uric acid in the total nitrogen ranging from 30-82%, allantoic acid 3.83%, urea 6.46-10.4% and ammonia 5.6-17.3%. Ammonia is the product most evident when entering a broiler house and gives rise to the alkaline conditons in the litter but the carbon dioxide produced leads to micro-aerophilic conditions within the litter.

The action of these nitrogenous compounds may be considered both in respect to their direct action on salmonellas and in combination with other parameters in the litter. Preliminary experiments showed that uric acid inhibited salmonellas at 1-1.5% level while these bacteria could tolerate up to 30% of urea in the media. As these nitrogenous compounds are important constituents of litter the interaction of the inhibitory effect of uric acid and water activity together with urea and temperature will be studied (Experiments 9.14 to 9.15). In contrast allatoin acid is a minor constitutent and so will be considered only in relation to its inhibitory capacity (Experiment 9.9).

A previous experiment has shown that in general the effect of pH on the persistence of salmonellas is similar irrespective whether sodium hydroxide or ammonia were used to adjust the media (Experiment 9.5). However Turnbull & Snoeyenbos (1973) concluded that ammonia was in part responsible for the inhibition in poultry litter. Therefore an experiment was designed to determine the toxicity of ammonia ions per se on salmonellas (Experiment 9.10).

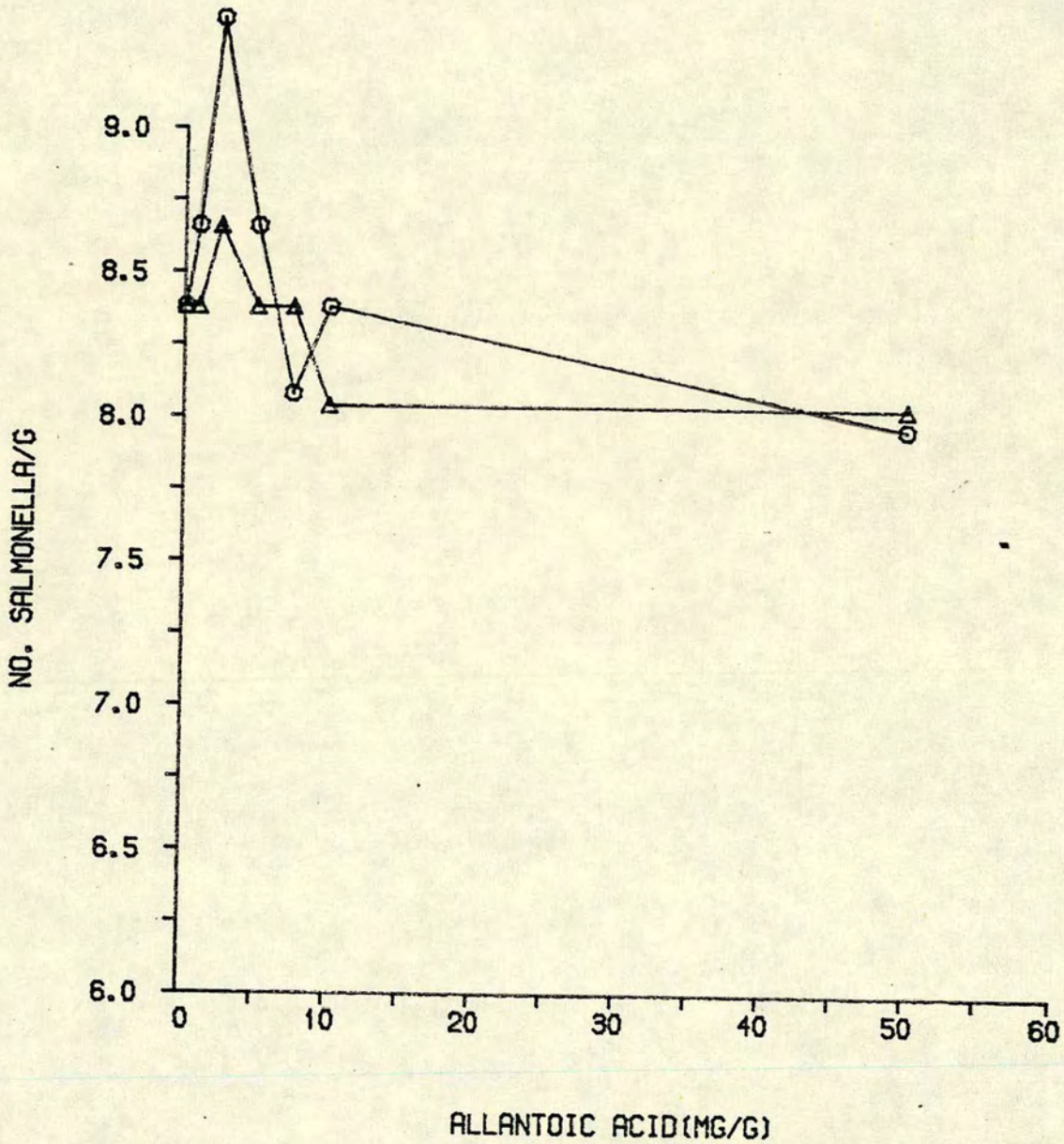
2. Experimental Work

(a) The Persistence of Salmonella typhimurium in Liquid Media containing Allantoic Acid (Experiment 9.9)

Allantoic acid was added to N broth and BP water at a range of concentrations from 0-100mg/ml. These solutions inoculated at the rate of 0.1ml of Salmonella typhimurium per 10ml quantity and incubated at 26°C for 2d the number of salmonella were determined by the stated

FIG. 9.9. THE PERSISTENCE OF SALMONELLAS IN THE PRESENCE OF ALLANTOIC ACID.

(KEY: ○ NUTRIENT BROTH, △ B.P. WATER.)



MPN-3 method.

Results

Allantoic acid even at 100mg/ml did not markedly inhibit salmonellas in N broth or BP water (Fig. 9.9) and as this product is only found transiently in litter it would appear to have little significance in the inhibition of salmonellas.

(b) The Persistence of Salmonella typhimurium in the presence of Ammonia Ions (Experiment 9.10)

Experimental Details

Solutions of ammonia salts which are incorporated in laboratory media were prepared at 2 molar strength and added to N broth and M_9 broth to give a range of concentrations from 0.001M to 1.5M concentrations.

Ten ml quantities of these solutions were then inoculated with 0.1ml of a 10^{-1} dilution of an overnight culture of S. typhimurium and then incubated at 26°C for 2d. The number of salmonellas was estimated by the MPN-3 method, the pH of the solution was recorded, and the presence of free ammonia ions estimated by mixing equal quantities of Nessler's Reagent and the test solution.

At the end of the of the incubation period the solutions with no visible growth were added to 100ml of BP water, which were incubated for 2-3d at 37°C to establish if the chemical additive was bacteriostatic or bacteriocidal.

Table 9.9

The Persistence of *Salmonella typhimurium* in the Presence of Equi-molecular Quantities of Ammonia Salts and Ammonia Solution (raw data in Table A9 (vi) in Appendix 2)

Chemical	Persistence in Salt of molar strength in M_9 Broth		N Broth		pH of 2M solution
	Inhibition	Toxicity	Inhibition	Toxicity	
Ammonium acetate	0.1	0.5	0.5	0.5	6.5
Ammonium chloride	0.1	>0.75	0.5	>0.75	6.0
Ammonium nitrate	0.1	0.5	0.5	>0.5	6.2
Ammonium phosphate	0.5	>0.75	<0.75	>0.75	5.1
Ammonium ortho-phosphate	0.5	>0.75	0.5	>0.75	7.6
Ammonium sulphate	<0.2	>0.2	0.5	>0.75	6.5
Ammonia solution	0.1	0.1	0.05	0.05	10.9

Results and Discussion

The addition of ammonia solution resulted in solution with high pH levels and the inclusion of equi-molecular weights of the salts resulting in solutions which were in the neutral range of pH values (Table 9.9). The presence of free ammonia ions were detected in all solutions at the end of the growth period.

There are substantial differences in the degree of ionisation of the salts so that the available ammonia would not be necessarily the same in all solutions. Both the basal media used had sufficient ammonia present to allow growth of bacteria and so increased level of ammonia would be expected to demonstrate a reduction of growth if ammonia ions are toxic.

The data detailed in Table A9 (iv) in Appendix 2 and summarised in Table 9.9 shows that high levels of the ammonia salts are required for toxicity, on the other hand a very low concentration of ammonia was toxic.

This experiment has shown that the addition of ammonia solution to laboratory media result in a rise in pH which in turn prevents the growth of S. typhimurium however the presence of ammonia ions as in the form of equi-molar concentration of ammonia salts did not have the corresponding effect. From these results it may be concluded that the pH level was the factor inhibitory to salmonellas and not the presence of ammonia ions per se.

I Feed Additives

1. Introduction

In addition to the nutritive ingredient of feeds small quantities

Table 9.10

Active Agent in Additive to Poultry Feed Manufacturers

Ingredient	P_{25}	P_{50}	P_{75}	P_{100}	P_{150}	Units
Vitamins						final feed
Vit. A	12	10	10	8	10	mg/kg
D_3	5	3	3	2	3	mg/kg
E	20	15	10	5	8	mg/kg
Coccidiostats						
Bambermycin	3	3	3	3	3	mg/kg
Favomycin 40						
Narasin-Monteban 100 (1.4%)	70	70	70	70	70	mg/kg
Trace elements						
Iodine	2.1	2.1	2.1	2.1	2.1	mg/kg
Selinium	0.2	0.2	0.2	0.1	0.1	mg/kg
Copper	14	14	14	7	7	mg/kg
Iron	40	40	40	20	20	mg/kg
Magnesium	120	120	100	100	80	mg/kg
Zinc	25	90	66	66	66	mg/kg
Molybdenum	0	0	0	0	0	mg/kg

of an additive containing trace elements, vitamins and coccidiostats are often present. As has been discussed in the Introduction of this section some of these materials are excreted unaltered by the birds and so can accumulate in the litter. In order to determine if these compounds be involved in the inhibition of salmonellas in litter compounds used routinely in a local vertically integrated enterprise were screened. The active ingredients of the compounds are given in Table 9.10.

2. Experimental Work

(a) The Inhibition of Salmonellas by Feed Additives as determined by the Disc Method (Experiment 9 .11)

Experimental Details

Plates of N agar and BPW agar were separately seeded with Salmonella agona, S. typhimurium and S. worthington as described in the General Materials and Methods. Aqueous solutions containing 10 and 100mg/l of the listed feed additive were placed on the surface of the agars. The plates were incubated at 37°C for 24h.

Results

The feed additives did not inhibit the three serotypes of salmonellas tested.

(b) The Inhibition of Salmonellas by Feed Additives incorporated into Agar (Experiment 9 .12)

Experimental Details

One gram of each feed additive was added to 9ml of N agar and

BPW agar and the mixture poured on a sterile petri-dish. After the agar had solidified and dried a loopful of each serotype viz. - Salmonella agona, S. typhimurium and S. worthington were streaked on each plate and corresponding plates of N agar and BPW water containing no additives. The plates were incubated at 37°C for 24h.

Results

The three serotypes all grew vigorously on all plates.

Discussion

The feed additives did not inhibit the growth of the three serotypes under consideration. In these experiments higher concentrations of the feed additives than would accumulate in litter were used therefore it is unlikely these additives contributed to the inhibition of salmonellas in the litter.

J The Persistence of Salmonella typhimurium when inhibition is exerted by two parameters

1 Introduction

In most natural situations more than one parameter exerts an influence on the microbes. As the various parameters do not all affect the same mechanism in the cell the total effect cannot be assumed to be the sum of the parameters determined as individual items. In the literature there is no information on the parameters which are interacting in the litter, however the water activity, temperature, pH, uric acid and urea have all been shown to exert an effect when tested separately and so the persistence of S. typhimurium

in situations where more than one of the parameters is present will now be determined.

2 Experimental Work

(a) In Laboratory Media

(i) Water Activity and pH value and temperature (Experiment 9.13)

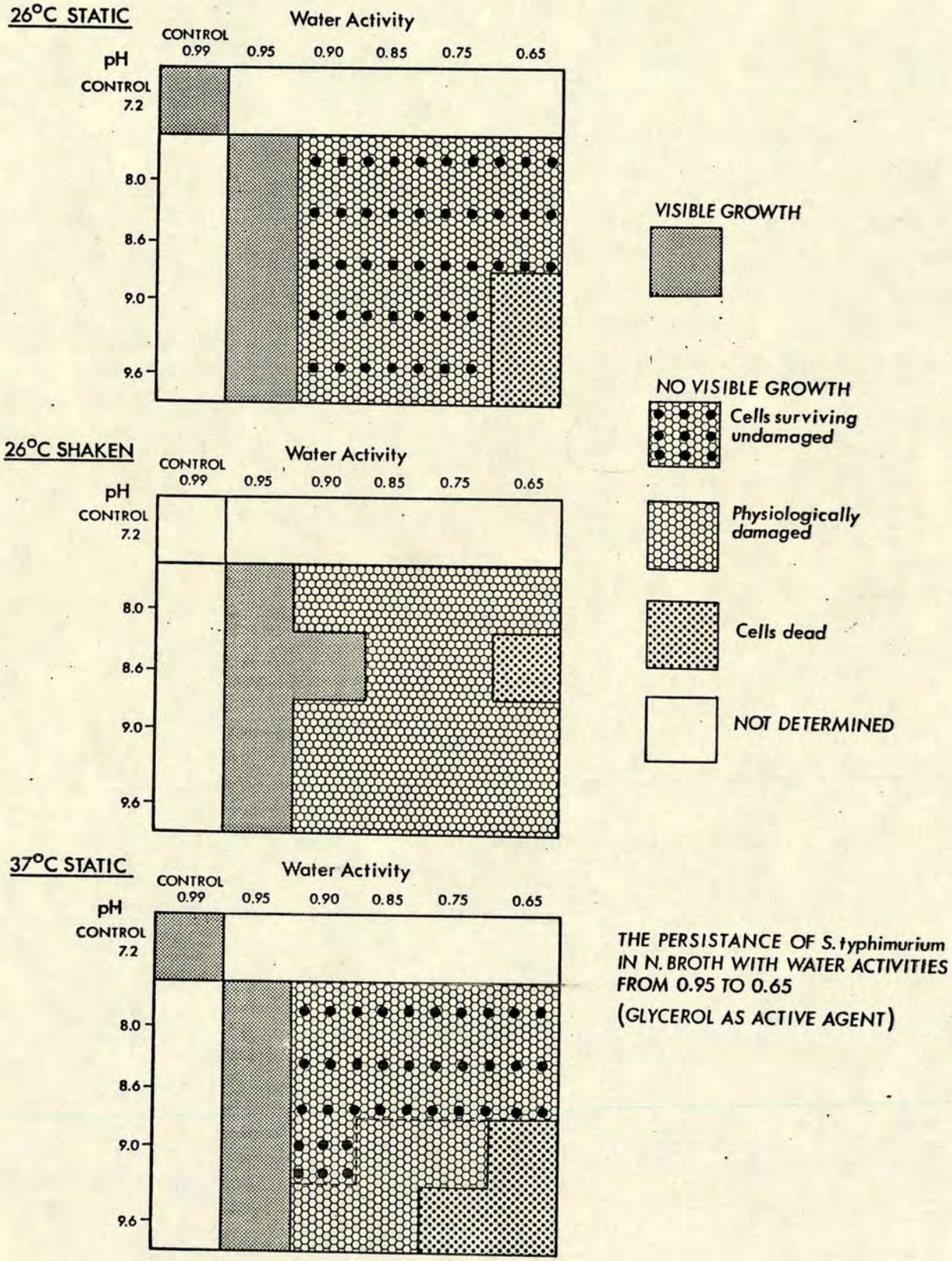
Preliminary experiments showed that sodium chloride and glycerol were both suitable for adjusting the water activity of N broth and M_9 broth. Similarly after investigation Tris-HCl buffer (Meynell & Meynell, 1970) sodium hydroxide, and potassium hydroxide were used to adjust the pH values of these broths, but sodium hydroxide was found to be the most suitable agent.

In the literature there is a great deal of information on the effect of acids on the survival of salmonellas in human foods so this investigation will concentrate on the alkaline pH values more prevalent in the litter.

Experimental Details

The pH value of N broth and M_9 broths were adjusted with 0.2M NaOH to give solutions with pH 8, 8.5, 9, 9.5 and 10. The water activity of the solutions adjusted over a range from Aw = 1.0 to Aw = 0.90 in the first series of experiment using glycerol or sodium hydroxide and Aw = 0.95 to Aw = 0.65 in the second series. Ten ml quantities of each solution was transferred to flawless test-tubes and inoculated with 0.1ml of 10^{-2} dilution of an overnight culture of S. typhimurium. The solutions were incubated statically at 26°C and 37°C and shaken at 26°C both for 72h, the turbidity being

FIG.9.10. INTERACTION OF WATER ACTIVITY & pH ON GROWTH OF SALMONELLAS.



THE PERSISTANCE OF *S. typhimurium* IN N. BROTH WITH WATER ACTIVITIES FROM 0.95 TO 0.65 (GLYCEROL AS ACTIVE AGENT)

read at predetermined intervals of time. The pH of each solution was recorded at the beginning and completion of the incubation period.

Those solutions showing no visible growth after 72h were further investigated to determine the condition of the surviving cells using the method given in the Materials and Methods section of this Chapter.

Results and Discussion

The data pertaining to the survival of S. typhimurium in N broth adjusted with glycerol over a range of Aw = 0.95 to Aw = 0.65 is shown in Fig. 9. 10 the results of the treatments vary only in detail from this pattern and are presented as raw data in Table A 9 (v) in Appendix 2.

An increase in alkalinity decreased the tolerance of salmonellas to a lowered water activity. When sodium chloride was added to N broth the bacteria were able to grow at Aw = 0.92 at pH 7.2 but only at Aw = 0.9 at pH 9 and Aw = 0.99 at pH 9.5. The levels at which growth was observed was identical for all incubation conditons. At Aw = 0.95 at pH 7.2 it was possible to culture salmonellas directly on BG agar but in all other solutions it was necessary to resuscitate the cells suggesting that the latter solutions damage but do not kill the cells while the former solutions are bacteriostatic.

In contrast in M_9 broth S. typhimurium at 26°C at pH 8 inhibition was found at Aw = 0.99 and death ensued at Aw = 0.94, the corresponding level at pH 9 being Aw = 0.98. However at 37°C when the cells were

multiplying more rapidly bacteria could not survive at Aw = 0.98 and pH 8. This demonstrates that under these conditions N broth is able to support growth under more extreme conditions than M_9 broth although salmonellas grow satisfactorily in both media at optimal conditions.

The addition of glycerol to N broth presents more exacting conditions, the growth patterns differing under the varying incubation conditions. For instance at 26°C in static conditions grow at pH 9.5 and Aw = 0.98 in shaken culture the level is pH 9.5 and Aw = 0.95 and at 37°C the corresponding level is pH 10 and Aw = 0.95.

In the second series of experiments the water activity was adjusted with glycerol from Aw = 99 to Aw = 0.65 which can be equated to material with moisture contents of over 50% to approximately 12.5% (Experiment 9.1). Under these conditions salmonellas grew up to pH 9.5 at Aw = 0.95 in all solutions. In the shaken cultures all cells exposed to lower water activities were damaged but death only ensued at Aw = 0.65 and pH 8.6. In contrast death took place at the higher pH levels at Aw = 0.65 at 26°C and 37°C (pH 9.0 at 26°C; pH 9.0 at Aw = 0.65; pH 9.6 Aw = 0.75 at 37°C) but fewer cells were damaged in static culture (26°C - none were damaged, 37°c pH 9.0 Aw = 0.9, pH 9.6 Aw = 0.90). In M_9 broth where the nutritional conditions were more exacting the cells were more easily damaged, undamaged cells only occurring at Aw = 0.90, death took place at pH 9.0 in static solutions. However in the shaken solution survival was found at pH 10.

These results show that at low levels of water activity in alkaline conditions the limit of survival depends greatly on the medium and

rate of aeration. Therefore it is impossible to define conditions which would inhibit salmonellas and could be related to conditions within litters. However the higher the level of alkalinity the lower the moisture content the greater the possibility that salmonellas will not survive. These results do illustrate that salmonellas can survive fairly hostile conditions in nutrient solutions which will account for the continued presence of salmonellas in low numbers in some materials after contamination.

(ii) Water Activity and varying concentrations of Uric Acid (Experiment 9.14)

Experimental Details

A series of solutions containing known concentrations of uric acid were prepared in N broth and the water activity of 50ml quantities of each solution adjusted with glycerol to a range of water activities. Salmonella typhimurium was added at the rate of 0.1ml of a 10^{-3} dilution of an overnight culture to 10ml of broth and these solutions at 26°C for 2d.

After incubation the number of salmonellas was determined by the stated MPN-3 method.

Results

In N broth uric acid was toxic at 1% but as can be seen in Table 9.11 a decrease in water activity increased the toxic effect.

FIG.9.11. THE PERSISTENCE OF SALMONELLAS IN NUTRIENT BROTH CONTAINING VARIOUS CONCENTRATIONS OF UREA.

(INCUBATION CONDITIONS: o 26 C STATIC, Δ 26 C SHAKEN, ▽ 37 C STATIC)

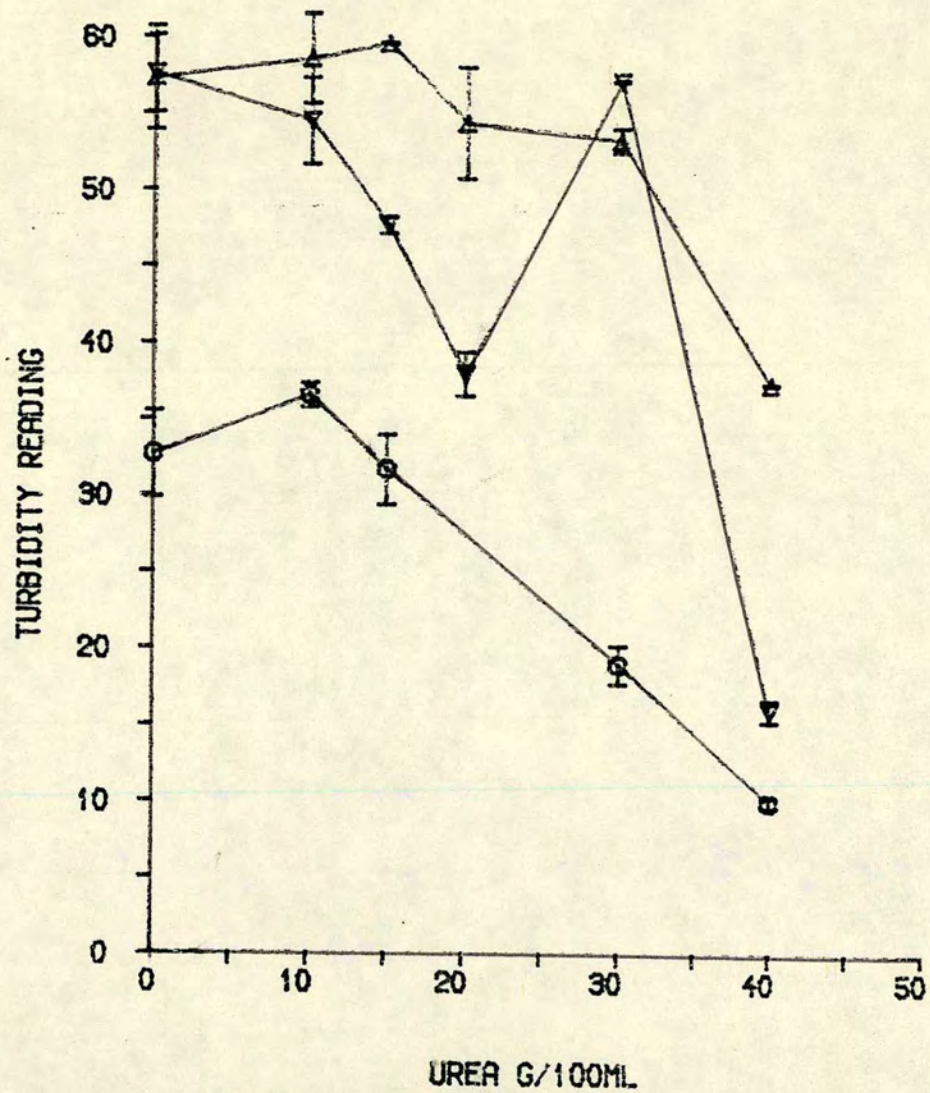


Table 9.11

The Persistence of Salmonella typhimurium in N broth containing a Range of Concentrations of Uric Acid at various levels of Water Activity

Water Activity	Number of salmonellas at Concentration of Uric Acid (%) 0.8	1.0	1.4
0.98	1.04	0.6	<1.0
0.96	1.88	<1.0	<1.0
0.94	<1.0	<1.0	<1.0

(iii) Varying Concentrations of Urea at three temperatures (Experiment 9.15)

Experimental Details

A solution of N broth containing 40% (w/w) of urea was sterilised by Seitz filtration. The solution was then diluted with a solution of N broth (sterilised) to give a range of concentrations of urea from 0% to 40%. Twelve 10ml quantities of each solution was inoculated with 0.1ml of 10^{-3} dilution of an overnight culture of S. typhimurium and three replicates incubated at 16°C, 26°C and 37°C statically and 26°C shaken in a water bath.

Turbidity readings were taken at 12h intervals to 72h. At 72h the pH level was determined for each solution.

Results

The pH of the solution remained in the range pH 6.85 to pH 7.85 and therefore will not have contributed to the inhibition of salmonellas (Fig. 9.11). In the shaken culture the logarithmic

phase was longer than in the corresponding static cultures but the total level of growth was greater. There was little growth at this temperature until 60h. The corresponding time for the visual assessment of growth was 12h at 37°C, 24h at 26°C incubated statically and 36h when shaken.

(b) The Persistence of Salmonella typhimurium in Litters with Parameters modified in the Laboratory

While experiments using laboratory media can give an indication of the possible performance of the salmonellas in litter such experiments cannot take into account the unique nature of poultry litter. The following three experiments will examine poultry litter under conditions when some parameters are modified.

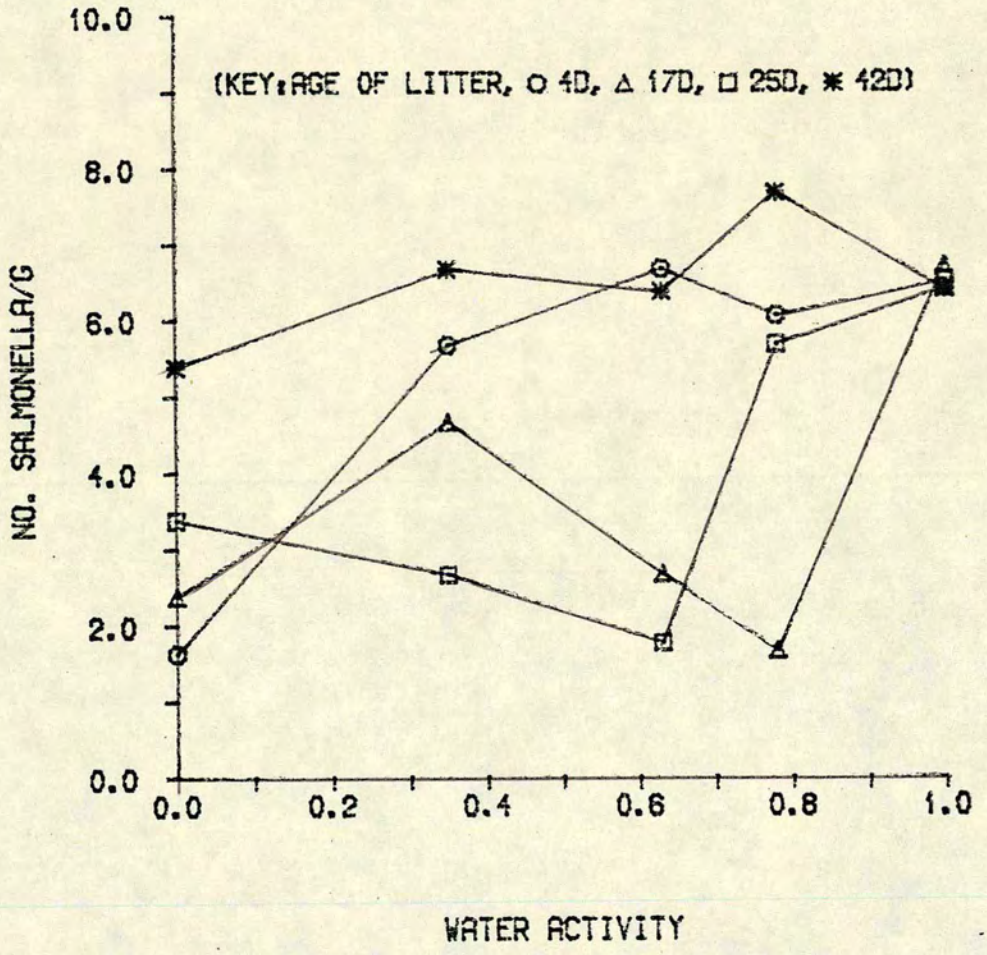
(i) Water Activity and Age of Litter (Experiment 9.16)

The visual changes in poultry litter during maturation is reflected in the chemical composition and microflora. This experiment will examine the possible effect of the changes on the growth of S. typhimurium when the moisture content is adjusted by exposing the litter to environments with five water activities.

Experimental Details

Litter of 4d, 17d, 25d, and 35-42d (L 37-40) were collected from poultry houses, stored for 18h at 4°C after which 1g quantities of each litter were allowed to stabilise at Aw = 0, 0.3, 0.5, 0.7 and 1.0 for 3d. The samples were then inoculated with 0.1ml quantities of 10^{-4} dilution of S. typhimurium and storage continued

FIG.9.12. THE PERSISTENCE OF SALMONELLAS IN LITTERS OF VARIOUS AGES WHEN WATER ACTIVITY WAS CONTROLLED.



for 2d when the number of salmonellas was determined using the stated MPN-3 method.

The characteristics of the litter at the commencement of the experiment is shown in Table 9.12.

Table 9.12

The Characteristics of Litter to be exposed to a Range of Water Activities (examined at 24h after sampling)

Litter Number	Age of Litter (d)	Moisture content (%)	pH	Survival of Salmonellas/g*
L 42	4	37.9	6.0	9.66
L 43	17	17.0	7.25	3.38
L 44	25	22.7	7.60	3.38
L 45	35-42	31.3	8.75	<1.00

*Inoculum 4.38/g

Results and Discussion

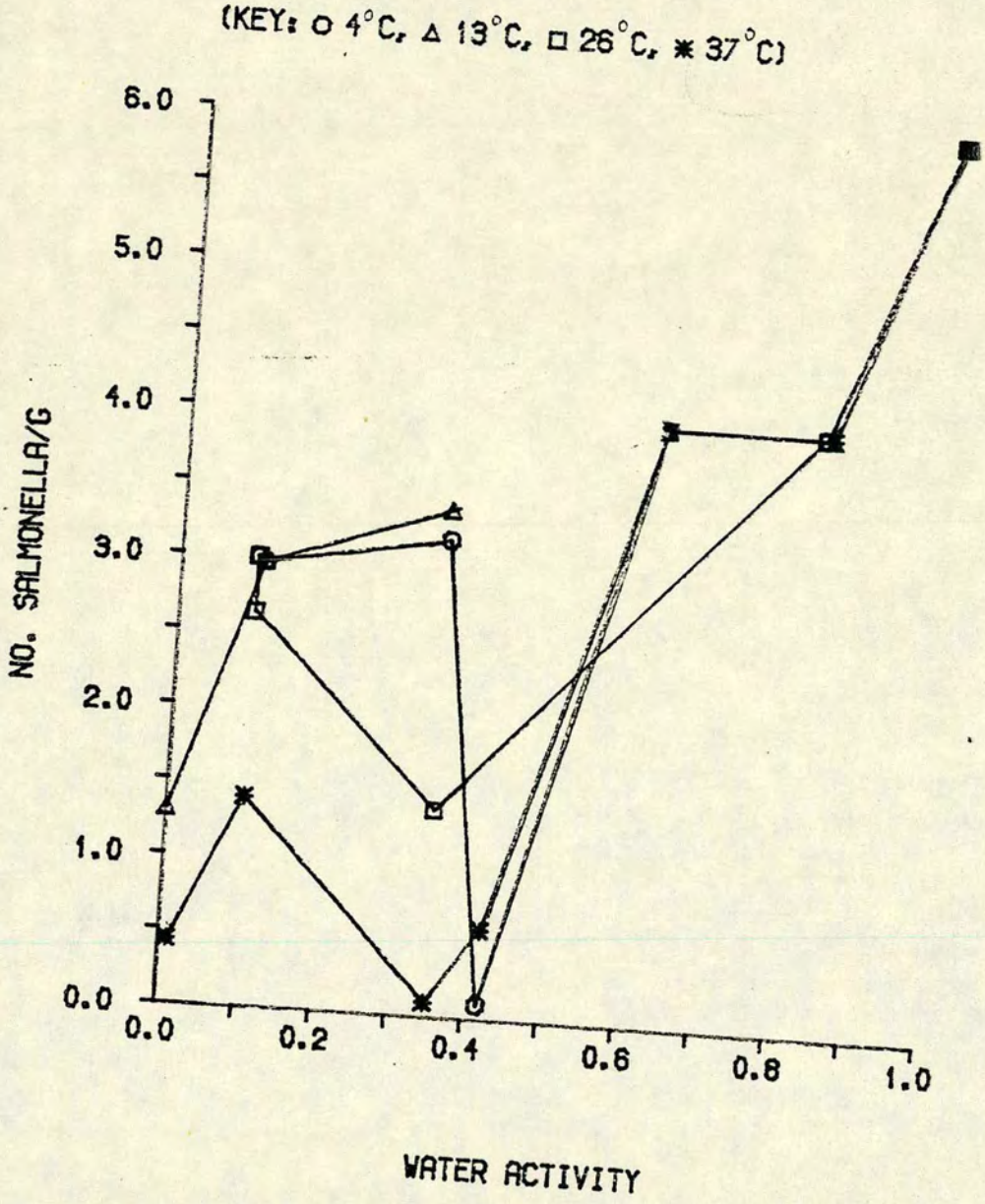
The results showed that the litter of all ages conformed to the pattern already established, the depression of persistence being at Aw = 0.5-0.6 (Fig. 9.12). The results of these experiments shows that the effect of water activity on the persistence of salmonellas is independent of age of the litter. It is noteworthy that salmonellas persisted in the litter of 35-42d equally as well as in the less mature litters.

(ii) Water Activity and Temperature (Experiment 9.17)

Experimental Details

Replicate 1g quantities of poultry litter (L 42) originally

FIG. 9. 13. THE PERSISTENCE OF SALMONELLAS IN LITTER STORED UNDER CONTROLLED CONDITIONS OF WATER ACTIVITY AND TEMPERATURE.



with a moisture content of 34.4% were equilibrated to a range of relative humidities at 4°C, 13°C, 26°C and 37°c. The method described in Experiment 9.3 was followed. The number of salmonellas persisting being estimated by the stated MPN-3 method.

Results

The temperature did not affect the number of salmonellas isolated at each relative humidity. (Fig 9.13)

(iii) Water Activity and pH Values (Experiment 9.17)

Experimental Details

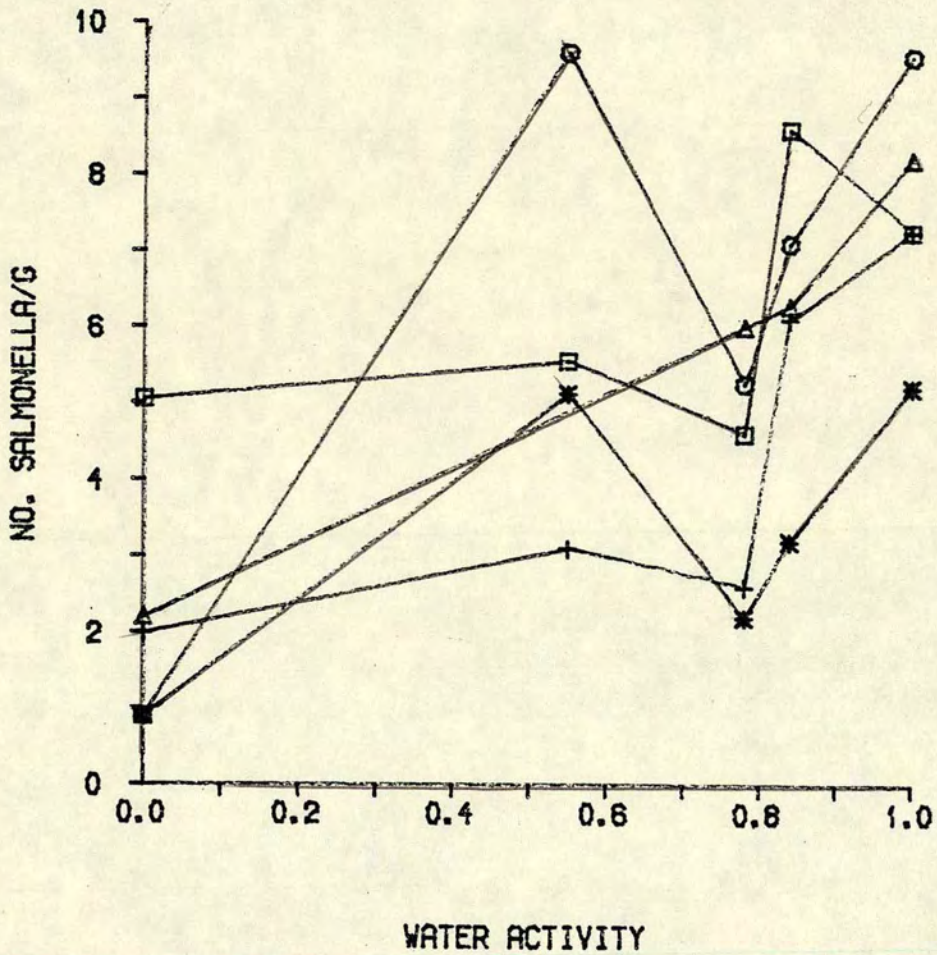
A litter of 27d old (L42) was taken and the pH of sub-samples of the litter adjusted to pH 4, 5, 6 and 10 with 2M HCl or 2M NaOH and the water activity of these "litters" stabilised over saturated solution of salts for 3d. The litters were then challenged with S. typhimurium as described in Experiment 9.3 and the number of salmonellas determined by the stated MPN-3 method after incubation at 26°C for 2d. The pH of the litter at the end of the experiment was also noted.

Results and Discussion

The data generated can be considered in two ways as the numbers of salmonellas recovered from the litters can be compared to the original pH of the litter or the final pH. When the water activity of the litter decreased so the persistence of the salmonellas declined, however the decline was less in the control samples indicating

FIG.9.14. THE PERSISTENCE OF SALMONELLAS IN LITTERS WITH PH VALUE AND TEMPERATURE. CONTROLLED.

(KEY: ○ PH 4, △ PH 5, + PH 6, □ PH 8.7, ✱ PH 10)



that the alteration in the pH levels had in some way modified the microflora of the litter or the chemical parameters in some manner during the period of equilibrium. When the number of salmonellas recovered at each pH level are considered in relation to the water activity it will be seen that there is a depression of numbers at AW = 0.1 and Aw = 0.78. The depression of growth at Aw = 0.78 is higher than found in previous experiments (Experiments 9.3, 9.4, 9.17 and 9.18).

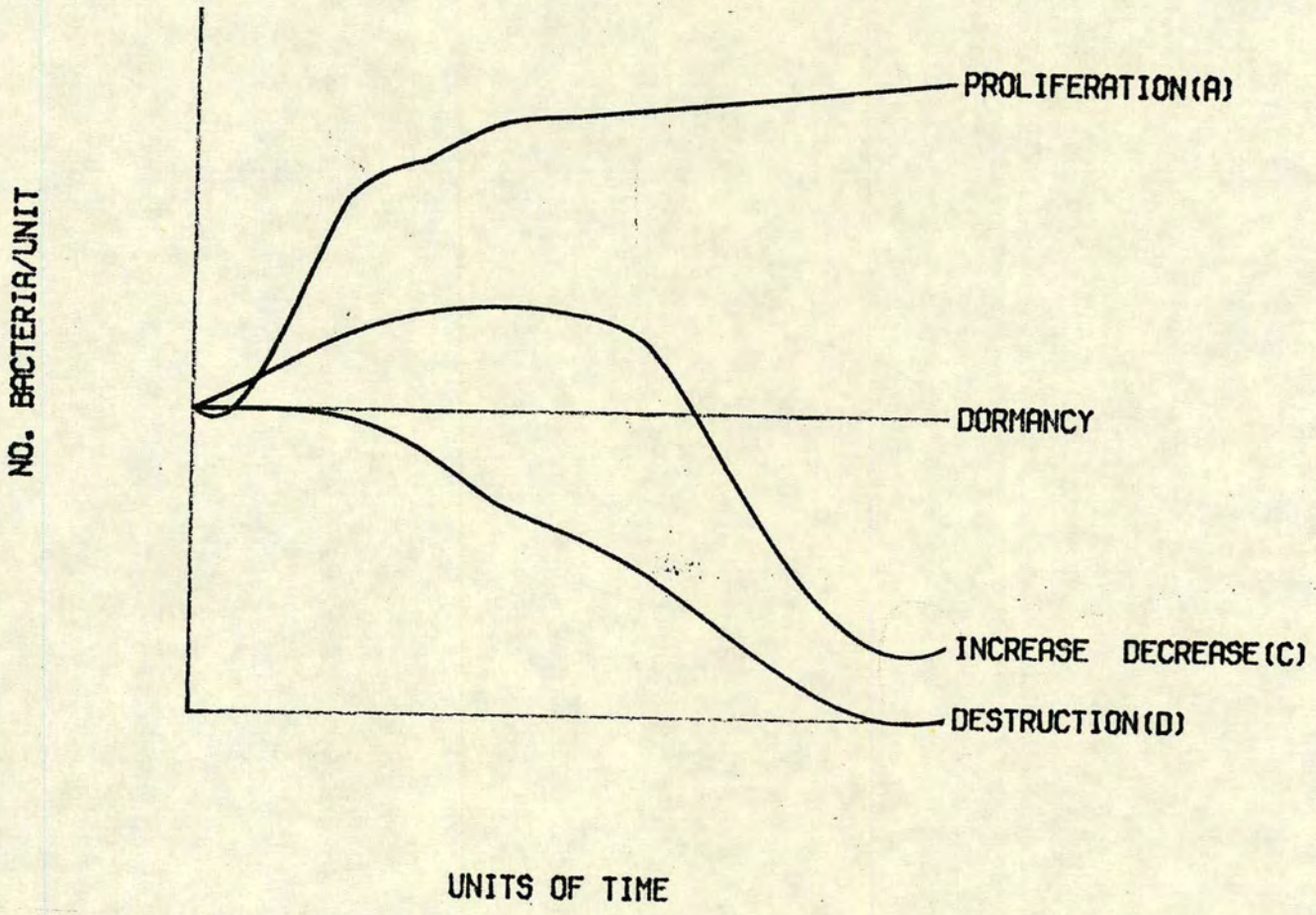
During storage the pH level tended to adjust towards a neutral pH. Fig. 9.14 shows the numbers of salmonellas isolated plotted against the pH value at 2d. As before a water activity of Aw = 0.1 did not allow the growth of salmonellas except in the sub-sample when the pH was not adjusted. However in general the pattern is very similar to that seen using initial pH except that the pH range has become compressed. The growth of salmonellas was favoured at pH 7.8.

Except for those litters maintained at Aw = 0.1 where there would be little bacterial activity the changes of pH during the incubation period was not related to the water activity in environment.

(K) DISCUSSION

Before considering the inhibition of salmonellas in litter it is necessary to define the patterns of persistence of bacteria as illustrated in Fig. 9.15. The curve representing proliferation (A) is the logarithmic pattern of growth quoted for bacteria in

FIG.9.15. THE THEORETICAL MODES OF GROWTH OF BACTERIA.



laboratory media and other situations of physiological affluence The initial log phase depends on the time taken for the cells to adjust to the new environment, the physiological state of the cells, previous treatment and growth conditions. Destruction (D) takes place when the rate of death is greater than the rate of multiplication. Mossel (1982) has suggested the addition of an extra line (C) to the classical curve of Monod-Hinshelwood where there is an initial increase followed by destruction. This growth is seen in materials where conditions initially favour growth but the presence of an inhibitor or a build-up of metabolic products adversely affect the vulnerable actively dividing cells. Except in the genera of bacteria forming spores a situation when the number of bacteria remains stable it is assumed that this is achieved when the multiplication rate balances the rate of destruction. There is however increasing evidence that a state of dormancy (D) present in non-sporing bacteria (Dow, 1983).

In complex material such as litter the salmonellas could be growing in the manners represented by all three curves in different micro-environments in the material. In addition a slight change in any of the conditions could modify the growth patterns from one mode to another. Therefore the change in numbers of salmonellas in litter represents a total change in numbers in the material and may not be representative of all sites in the litter.

In the early stages of the formation of litters the amount of faeces deposited is so small that there is unlikely to be sufficiently high concentrations of VFA's to exert an inhibitory

effect on salmonellas. Once the litter has become alkaline although the level of these compounds may have reached a critical level the pH level would preclude ionisation so eliminating the possibility of an inhibitory effect.

While uric acid, the main nitrogen excreta of the bird can exert an inhibitory effect *per se* if accumulated up to the 1% level, the effect exerted by the breakdown products would appear to be a major factor in inhibiting salmonellas. As already shown uric acid breaks down to ammonia and carbon dioxide with allantoic acid and urea as intermediates. This investigation has shown that allantoic acid plays no part in the inhibition of salmonellas. Similarly urea needs to be present at levels much higher than those found in litter to be an effective inhibitor however urea is able to reduce the water activity and so contribute to this parameter. The accumulation of ammonia in the litter leads to an alkaline pH which is an important factor as even in laboratory media the number of salmonellas decreases at pH values over pH 8.0. In materials with a pH value in excess of pH 8.5 that rate of decrease is $0.33 \log_{10}$/0.1 unit increase in pH value.

In this investigation it has been shown that the influence of ammonia is due to the change of pH value which it causes rather than the effect of the ammonia ions *per se*. This conclusion suggests that any alkaline compound could be added to achieve this effect, which could be used as a method of controlling salmonellas in litter under young chicks as an aid to controlling salmonellas in the flocks.

The production of carbon dioxide in the litter will lead to conditions which favour the multiplication of salmonella.

Water activity within the litter exerts a powerful effect on the prevention of growth of salmonellas. At the high levels of water activities (over Aw = 0.95) there is some multiplication of these bacteria while at the opposite end of the scale (Aw = 0.1-10) the cells may be "dormant". In the middle of the range there is a notable inhibition. This is possibly due to the death of cells which had been previously stimulated into growth, for actively metabolising cells are more vulnerable to inhibitors. The greatest inhibition was found to vary in these experiments from Aw = 0.35 to Aw = 0.50 which would represent moisture contents in materials of 7% to 10% although in one experiment the decline in numbers was at Aw = 0.75 (12.5% moisture content being the equivalent water activity). Therefore it may be concluded that litters with moisture contents of 5-15% moisture content have a level of activity which promotes the inhibition of salmonellas.

In litter no physico-chemical factors act in isolation. While laboratory experiments do not represent the conditions in litter the effect of the interactions can be more easily studied by this method. Using an undefined medium (N broth) it has been shown that an increase in alkalinity reduces the tolerance of salmonellas to a lowered water activity. This effect is more marked in a defined medium (M_9 broth) emphasising that under adverse conditons the metabolic pathways used differ leading to nutritional changes more easily satisfied by the complex media. No growth was shown in media with

low water activities but salmonellas were found to survive at values as low as Aw = 0.65 in N broth but Aw = 0.85 in M_9 broth both at pH 8.5. When these effects were compared to those found in litter it was found that the inhibitory effect was similar but there appeared to be another parameter exerting an effect so confusing the results. As this additional inhibitor can often be removed by autoclaving it may be concluded that this factor is due to bacterial activity and this will be discussed in the next chapter.

From these results it may be concluded that the inhibitory action exerted by physico-chemical parameters is mainly due to alkaline pH levels resulting from the breakdown of uric acid to ammonia together with a water activity optimal for the destruction of the cells.

SUMMARY

1. The persistence of salmonellas in the litter is not the sum of the individual inhibitory factors.

2. No single factor would appear to be responsible for the inhibition of salmonellas in litter.

3. The alkaline pH level and a water activity between Aw = 0.4-0.6 are the two most important parameters in the inhibition of salmonellas in litters.

4. There is circumstantial evidence that cells of salmonellas may be in a state of dormancy in adverse conditions.

5. Contrasting the results of experiments in laboratory media and litter suggest that in litter an additional factor, possibly bacterial activity, is exerting an effect.

CHAPTER X

THE EFFECT OF MICROBIOLOGICAL FACTORS IN THE PERSISTENCE OF SALMONELLAS IN LITTER. 1. THE MICROFLORA OF POULTRY LITTERS

THE EFFECT OF MICROBIOLOGICAL FACTORS IN THE PERSISTENCE OF SALMONELLAS IN LITTER. 1. THE MICROFLORA OF POULTRY LITTERS

A Introduction

In some experiments when litter is used as the basal media there have been indications that there was an inhibitory factor exerting an effect in addition to the parameters which the individual experiment was designed to study. It is possible that this is microbial activity which is investigated in the remaining chapters.

A review of the literature will show that there is little information on the numbers of the major groups of bacteria in litters. Therefore initially litter samples were collected from poultry houses and the numbers of the main groups of bacteria enumerated.

B Materials and Methods

1. Bacteriological Media

(a) Blood agar (DST agar + 7% Horse blood [SR50]).

(b) EYGA agar (Currie & Keddie, 1973).

(c) Glucose agar (N agar with the addition of 1% Glucose).

(d) Hippurate Medium (Cowan & Steel, 1974).

(e) Litmus Milk (Oxoid CM45).

(f) Nutrient gelatine agar (N broth with the addition of 1.5% gelatine (Oxoid CM135a).

(g) Peptone-yeast-extract-agar (Schefferle, 1965).

(h) Starch agar (N agar with the addition of 1% soluble starch).

C The Microflora of Poultry Litter on Commercial and Non-Commercial Units

(a) Review of Literature

The earliest description of the microflora of poultry litter was Rahner (1801) who isolated Bacillus coli-gallinarium and B. coli-commune which he considered to be derived from the intestine of the bird and Micrococcus sp. Bacillus mesentericus, B. megatherium, B. fluorescens and a few yeast and moulds contributed by the feed and air. In 1957 when the present system of broiler production was still novel in Great Britain Schefferle (1957; 1965) investigated the bacterial flora of litter with particular reference to the decomposition of urea and the production of Vitamin B_{12}. She found that mature litter yielded an aerobic content of 10.0-11.0 bacteria/g of which 5.0-7.0/g were enterococci, also include a Corynebacterium helvolum, C. ureafactiens, Nocardia sp., Streptomyces spp., Micrococcus pyogenes var. albus, M. flavus, M. epidermis, Alcaligenes viscosus, Flavobacterium solare, Cytophaga sp., Bacillium globiforme, B. lineus, B. ammoniagenes, Pseudomonas ovalis and other unidentified pseudomonads. More recently Alexander et al. (1968) who concentrating on potentially pathogenic bacteria isolated Enterobacteriaceae (not salmonellas), Bacillus sp., Staphylococcus spp. and Streptococcus spp. from all samples and Clostridium butyricum, C. chauvoei, C. cochlearium, C. histolyticum, C. multifermentans, C. novyi, C. perfringens, C. sordelli, C. tetranomorphum, Corynebacterium equi, C. pyogenes, Salmonellas blockley, S. saint-paul, S. typhimurium var. copenhagen, Actinobacillus spp. Mycobacterium spp. in a limited number of samples.

Table 10.1 continued

Species	Lovett (1977)	Dennis & Gee (1973)	Waldrip (1974)	Bacon & Bardick (1977)	Pinello et al. (1977)	So et al. (1978)
P. commune						X
P. chrysoqum		X			X	
P. courtosum		X				
P. cyclopium				X		X
P. decumber						X
P. funiculosum						X
P. herquei						X
P. lanorum						X
P. miezynskii						X
P. patulum				X		
Petriella setifora				X		
Pullalaria sp.	X					
Scopulariopis sp.	X	X				
Scop. brecicaulus				X		
Sepedonium sp.	X					
Thermococcus aurantiocus			X			
Trichoderma viride				X		
Trd. roseum				X		
Isolation Temperature	37°C & 22°C	37°C & 25°C	42°C			
pH	3.9-8.6	-	-	5.4-8.2		
Moisture content %	13.8-39.0	9°-26.0	-			12-40
Material	Wood Chip	Various	Wood Chip			Bark Residues

Table 10.1

Fungi isolated from Poultry Litter as described in Literature

Species	Lovett (1977)	Dennis & Gee (1973)	Waldrip (1974)	Bacon & Bardick (1977)	Pinello et al. (1977)	So et al. (1978)
Absidia cylindrospora		X				
Alternaric sp.	X					
Aposhceria sp.						X
Asperqillus sp.	X	X	X	X	X	X
A. amstelodomi		X				
A. candidus		X		X		
A. chevalieri						
A. clavatus				X		
A. flavus		X		X		X
A. fumigatus			X	X		X
A. qlaucus					X	
A. niger						X
A. petrakii		X				
A. restrictus		X				
A. ruber		X			X	
A. sydowi		X				
A. tonophilis					X	
A. versicolor		X				
Candida albicams	X					X
Cephalosporium sp.	X					
Ceph. roseum						X
Cladosporium	X	X		X		
Dactylaria cladosporium			X			
Fusarium sp.	X					
F. moniliforms						X
F. poa			X			
F. roseum						X
Geotrichium						X
Graphium putredinin				X		
Graphium pulredinin				X		
Heliocomyces sp.	X					
Hydodendron	X					
Microscus demonsporus				X		
Mic. larqirostra	X					
Mucor sp.	X			X		X
M. pusillus			X			
M. racemosus		X				
Oidioderdron fuscum				X		
Oidium sp.	X					
Oospora sp.	X					
Paecomycetes varioti		X	X			
Paecparvis		X				
Penicillium sp.	X	X		X	X	X
P. brevicomperilum		X		X		

All the workers who have enumerated the bacteria in litter agree that the numbers increase rapidly during the first few weeks. The rate of multiplication was found by Lovett et al. (1971) to be from 0.21 bacteria/g in unused wood chips to 7.0-8.0 bacteria/g at 7d old. They also found that mature litters had an aerobic count of 9.0 bacteria/g, anaerobic count of 9.0-10.0/g and coliform at 7.0-8.0/g of which 5.0-7.0/g were E. coli in addition to 0.6 fungi/g. Ivos et al. (1965) reported similar numbers (8.97 aerobic bacteria/g and 7.55 coliform/g). More recently Arafa et al. (1979) indicated that in the later stages of maturation there is a slight reduction in the number of total bacteria, the number varying from 9.0 aerobic bacteria in "young" litter and 8.0/g in older litter, the corresponding figures for anaerobic bacteria being 9.0 and 8.7/g. A similar pattern was found the Doerr et al. (1980) who found 6.55 bacteria/g in new litter, at 3 months the number was 8.63/g but at 6 months 7.54/g.

The mycological aspects of poultry litter has received more detailed attention than the bacteriology of this material. The number of fungi isolated have ranged from "very few" (Schefferle, 1957), 5.0/g (So et al., 1978), 5.0-7.0/g (Lovette et al., 1971) to 8.0/g (Carter et al., 1979). The latter workers (Carter et al., 1979) found that the numbers of fungi in the two series of litters which they examined differed in that while numbers in one litter remained within the range 7.0 to 8.0/g in the other the numbers declined. Doerr et al. (1980) also found a decline in numbers from 6.8/g to 3.74/g over 3 months after which the numbers remained fairly constant. The wide range of mould species which have been identified are shown in Table 10.1

This review of literature indicates that the microflora of poultry litter varies widely and has not been completely defined. The object of this investigation is to detect those bacteria which contribute either directly or indirectly to the inhibition of salmonellas in the litter. No attempt was made to fully identify the bacterial species encountered. It was however necessary to determine the dominant groups of bacteria in the litter through the life cycle of the birds when the inhibitory effect is developing.

D Experimental Work

(1) The Bacterial Flora of Litter of Various Ages from Two Poultry Enterprises (Monitoring Exercise 10.1)

In order to ascertain the relative numbers of bacteria in poultry litter maintained under the husbandry methods of the East of Scotland, samples were collected from broiler houses from two enterprises in the locality of Edinburgh.

Experimental Details

Litters of a range of ages were collected from two enterprises all the sampling being undertaken on one day. At Site A the sampling was carried out by the author while at Site B this was undertaken by the farm staff.

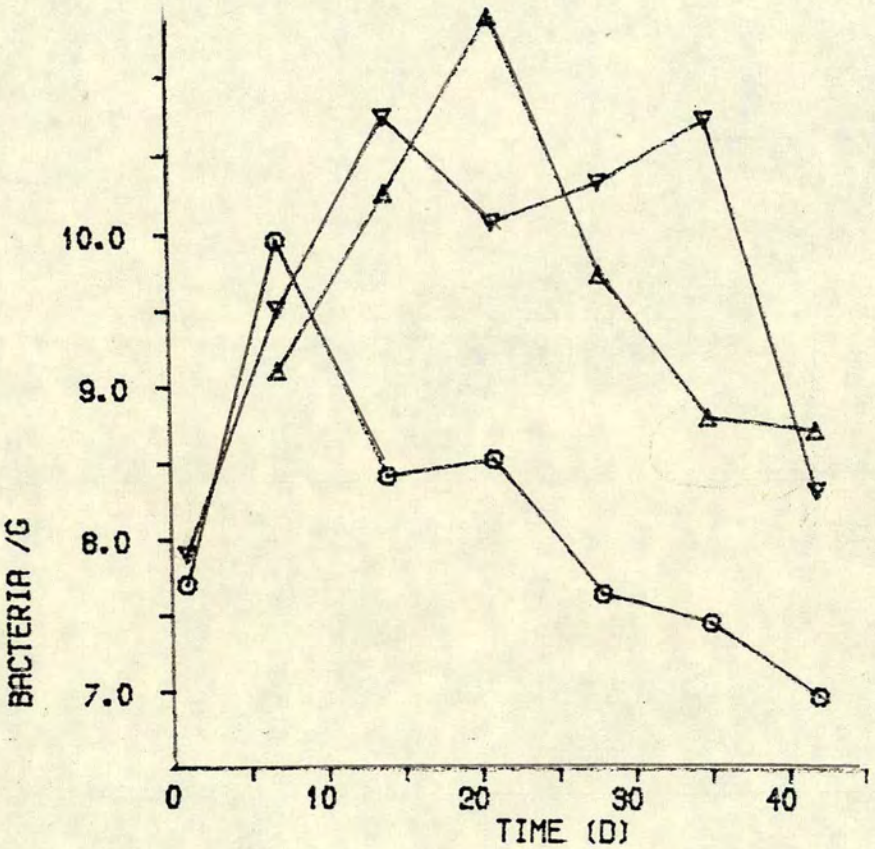
The following items were examined:-

1. Total bacterial count at 26°C on N agar and PC agar. with the addition of 10ml litter extract per litre.
2. Anaerobic bacterial count at 26°C on N agar.

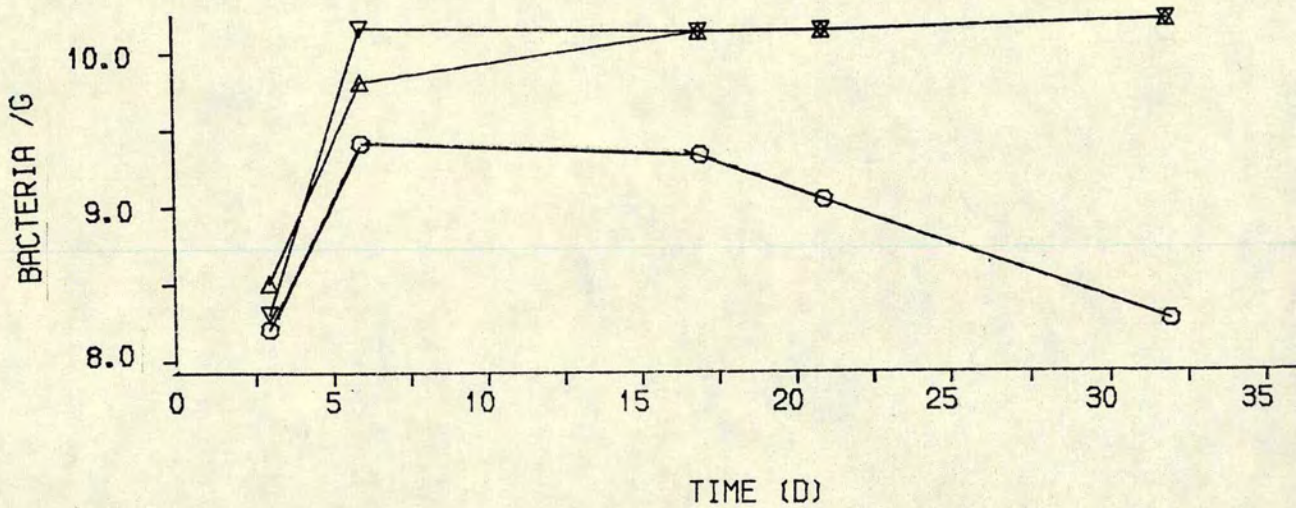
FIG. 10.1. THE MICROBIOLOGICAL ANALYSIS OF LITTER FROM TWO POULTRY ENTERPRISES.

(KEY: ▽ VL AGAR, △ PC AGAR, ○ ANAEROBIC N AGAR)

(A) THE BACTERIAL FLORA OF ENTERPRISE A



(B) THE BACTERIAL FLORA OF LITTER AT ENTERPRISE B.



3. Urea decomposing bacteria by MPN-3 method using urea decomposing broth.

4. The persistence of salmonellas determined by inoculating 1g of each litter with 0.1ml of 10^{-4} dilution of an overnight culture of Salmonella typhimurium. The numbers of the bacteria surviving after 2d incubation at 26°C being determined by the stated MPN-3 method.

5. Moisture content.

6. pH value.

7. The level of ammonia was determined by the accurate method (Chapter 2 D 2c.)

Result and Discussion

Large numbers of bacteria were isolated from all the samples on each media and both atmospheres used for culture (Fig. 10.1a and b) At Enterprise A except for the sample from a house containing birds of 3d all litters had about 10.0 bacteria/g, while at Enterprise B the sample had 8.5 to 11.5 bacteria/g. This could be due to the sampling method and storage at Enterprise B where the staff undertook this task. At both enterprises the urea decomposing bacteria numbered 7.0-9.0/g.

Although the numbers of bacteria in each sample remained fairly constant the pH value was higher in the older litter and similarly the ammonia content was 0.002mg/g in the earlier litters but 5.2mg/g in the litter of 42d (Fig. 10.2).

The litter of more than 30d were inhibitory for salmonellas (Fig.10.3) this inhibition being correlated with the age of the litter

FIG.10.2. THE AMMONIA CONCENTRATION IN LITTER IN A COMMERCIAL HOUSE.

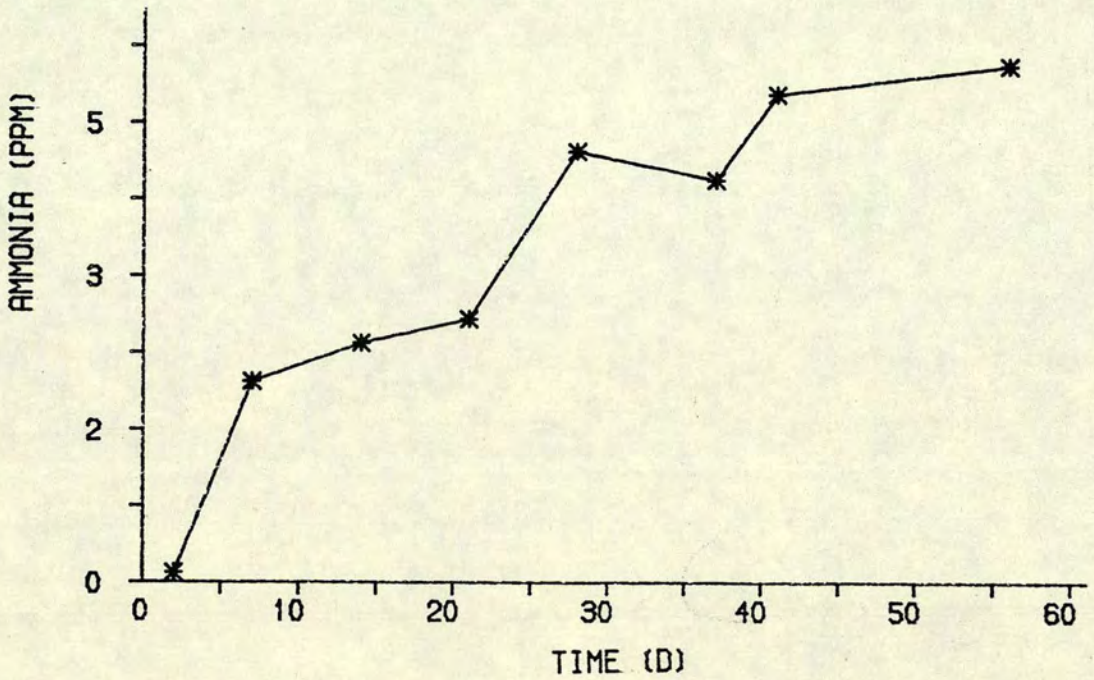
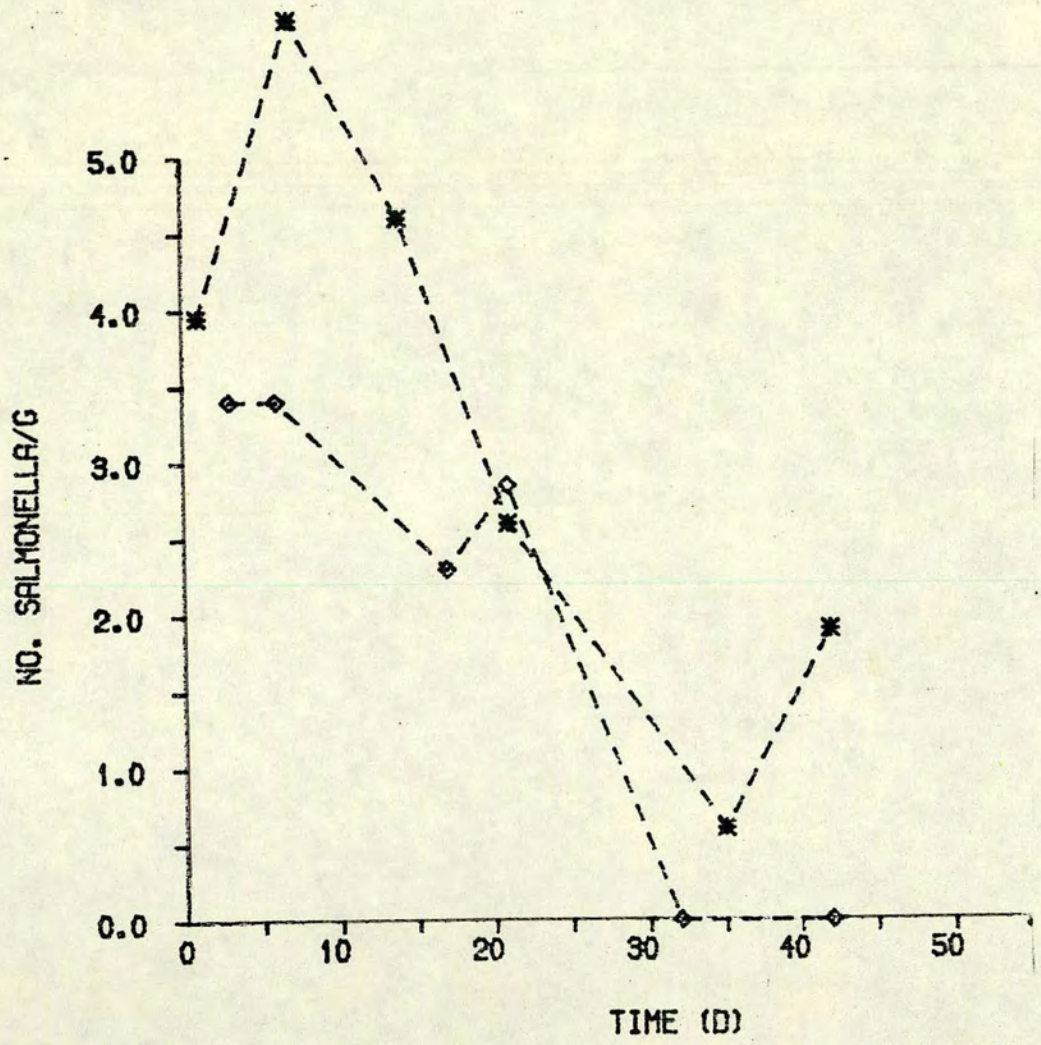


FIG.10.3. THE PERSISTENCE OF SALMONELLAS IN LITTER FROM COMMERCIAL ENTERPRISES.

(KEY, ◇ ENTERPRISE A, ✱ ENTERPRISE B)



(86% level). The pH value was similarly correlated to the inhibitory function of the litter but there was no correlation between moisture content and inhibition.

These samples were all taken from different houses and so it is possible that the variation could be dependent on the individual management of the houses rather than a real difference due to the age of the litter.

2. The Bacterial Flora of Litter from a Commercial Poultry House sampled at Weekly Intervals (Monitoring Exercise 10.2)

In the previous trial the sample was drawn from different houses so could not be said to be directly related although they were all managed according to the same management programme. However these results had shown trends which are worthy of confirmation by the analysis of serial samples from a single house. Therefore litter samples were collected from a commercial poultry house at weekly intervals throughout the 42d rearing period. In order to gain some insight into the succession of bacteria additional selective media were used to pin-point the growth of those groups of bacteria which could be involved in the inhibition of salmonellas in the litter.

Experimental Details

At regular intervals representative samples were taken from the whole house using the method described in the General Materials and Methods (2. B). Samples were taken immediately to the laboratory, the analysis commencing within 2h of collection. Preparation of the plates for incubation under anaerobic condition was completed

within 15 mins and the entire inoculation carried out within 24 mins. Freshly prepared media was used on all occasions.

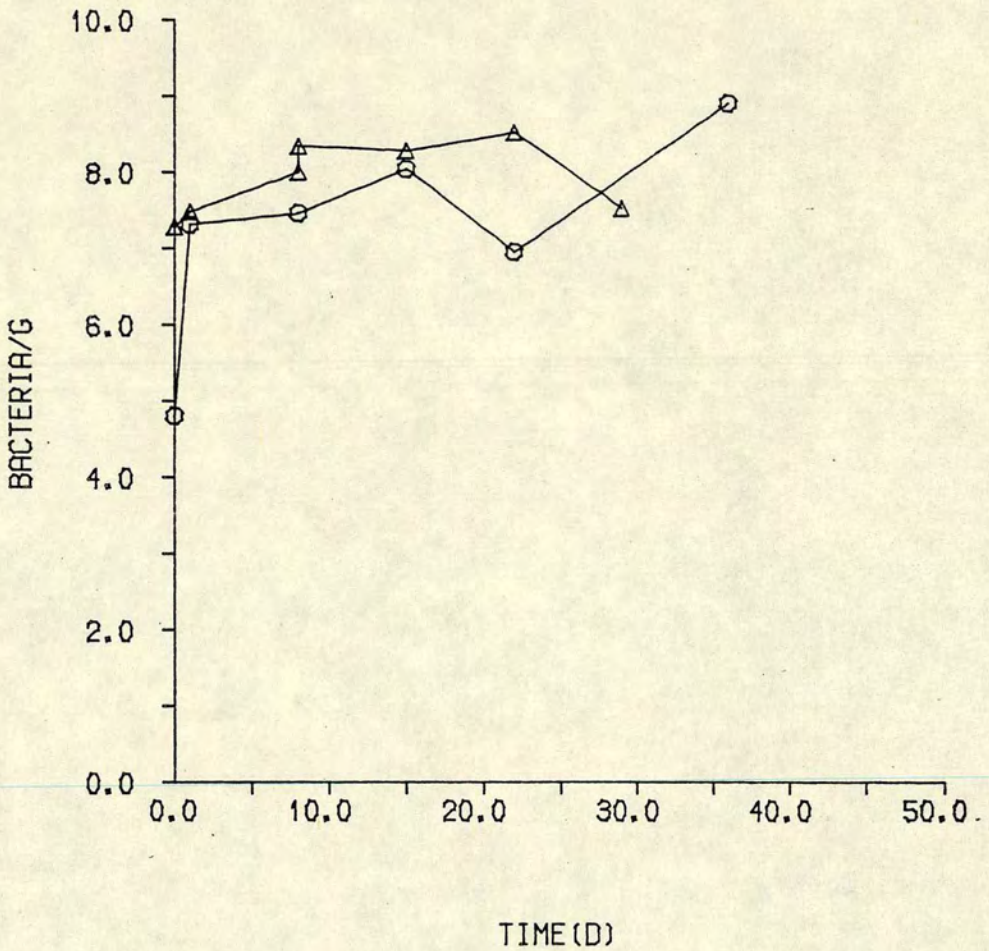
The initial dilution of the samples was made by adding 10g litter to 90ml RCM broth containing 5-6 marbles, the suspension being carefully mixed to ensure that the smaple was not aerated. Further decimal dilutions were carried out in RCM broth, 20ml of diluent being prepared at each level (Impey, pers. comm.).

The media, gaseous environment, incubation times and temperature were as follows:-

1. Total bacteria count VL agar, VLhfl agar, HI agar, aerobically and anaerobically at 26°C and 37°C.
2. Uric acid degrading bacteria - Uric acid degradation agar at 26°C and 37°C aerobically and anaerobically.
3. Urea decomposing bacteria - Urea decomposing broth at 26°C aerobically and anaerobically.
4. Lactic acid bacteria - Rogosa agar incubated at 26°C and 37°C anaerobically.
5. Streptococci on TAT agar at 26°C and 37°C aerobically and anaerobically at 26°C and 37°C.
6. Cellulose decomposing bacteria using the method of Mann (1972) incubated aerobically at 26°C.
7. Coli-aerogenes bacteria - MacConkey agar at 26°C and 37°C incubated anaerobically.
8. Bacteriodes group of bacteria - EV agar and VK agar incubated anaerobically at 26°C.

FIG.10.4. THE BACTERIA IN LITTER FROM A COMMERCIAL HOUSE SAMPLED WEEKLY.

(KEY: ○ ANAEROBIC BACTERIA, △ AEROBIC BACTERIA)



9. Yeasts and moulds on OAES agar and malt extract agar incubated aerobically at 26°C.

10. Actinomycetes using ½ TSA agar and CZ agar at 26°C and 55°C incubated aerobically.

11. The persistence of salmonellas in the litter was assessed by inoculating 1g of the litter with 0.1ml of 10^{-4} dilution of an overnight culture of Salmonella typhimurium and the number of cells estimated by the stated MPN-3 method after incubation at 26°C for 2d.

12. pH value.

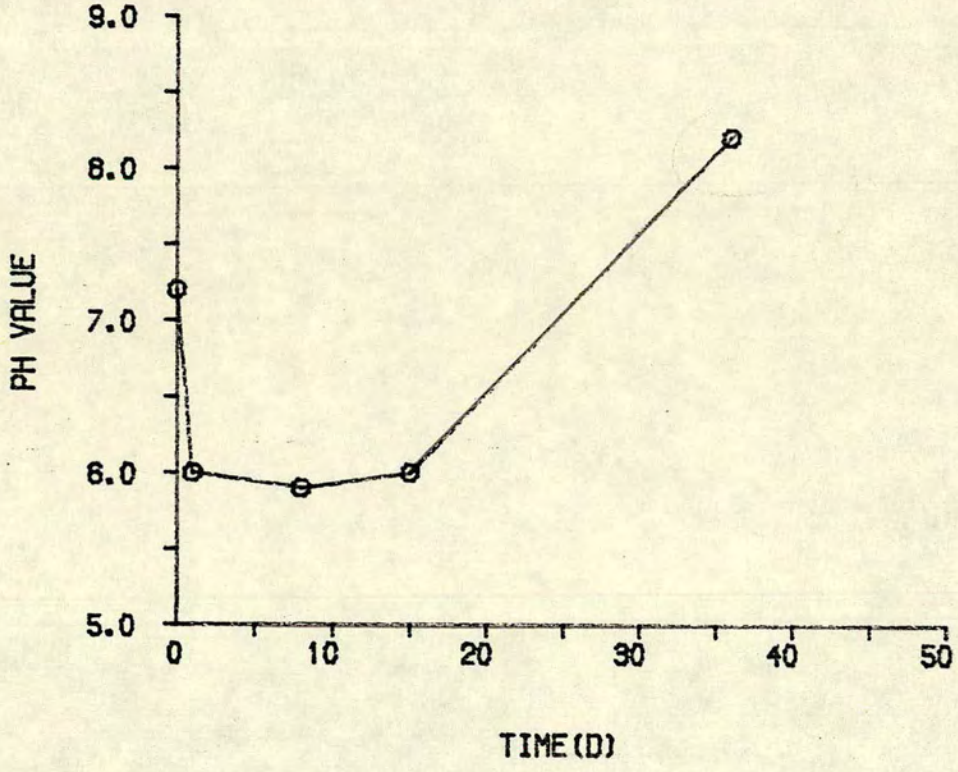
13. Moisture content.

Results and Discussion

Sequential sampling at a single house confirmed the trend suggested by the results of the samples collected from different houses (Monitoring Exercise 10.2). The total count rose in the first few days and then remained steadily at 7.0-8.0/g for the remainder of the rearing period. In this trial 5.0 bacteria/g were isolated on VLhfl agar on the day on which the chicks were placed, a higher number than would be anticipated in a fumigated litter. VLH agar was the most successful media for isolating bacteria at the early stages of rearing, as bacteria being cultured on HI agar using the identical dilution solutions for analysis.

The numbers of bacteria rose in the first 15d and stayed level for the next 15d after which the numbers decreased slightly (Fig. 10.4). The counts of lactic acid bacteria normally considered to

FIG.10.5. THE PH VALUE OF LITTER FROM A COMMERCIAL HOUSE SAMPLED WEEKLY.



be the group of bacteria first established in the chicken intestine (Ochi et al., 1964) rose rapidly in the first 10d and then decreased as the litter matured indicating that this group are not favoured by the conditions in the litter. In contrast the bacteria of the Bacteriodes* and coli-aerogenes groups, both found in the mature chicken gut flora became established in the litter at the level of 6.0-7.0/g after 3d. Uric acid decomposing bacteria and streptococci achieved their maximum number by the 20th day and then remained at a constant level for the remainder of the rearing period. Yeasts were isolated in higher numbers than had been seen in other samples examined at this laboratory, 2.0-5.0/g were isolated from the unused sawdust which when considered in conjunction with the bacterial results suggest that the fumigation had not been as effective in reducing the number of micro-organisms in the wood shavings as would have been anticipated.

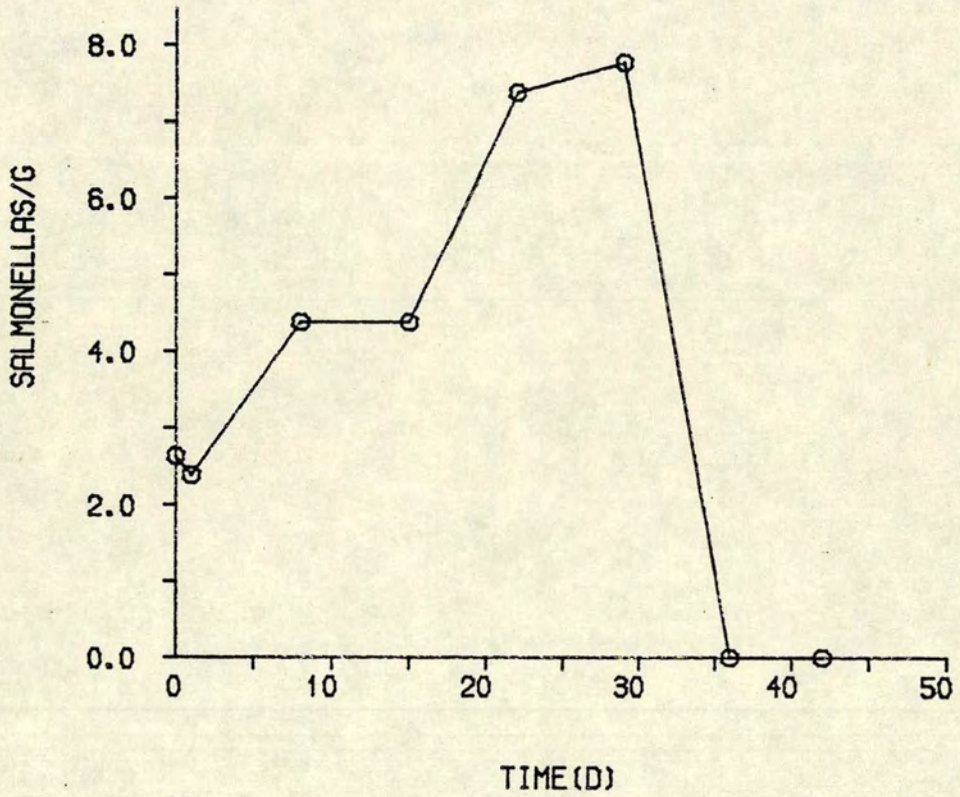
The number of cellulose decomposing bacteria, moulds and actinomycetes never exceeded 2.0/g at any stage of rearing.

The moisture content of the litter and the pH value gradually increased as the litter aged. As the pH reached a maximum level 7d after the number of uric acid decomposing bacteria reached a maximum level it is likely that the increased alkalinity is largely due to the production of ammonia by these bacteria. It is possible that the alkalinity could then limit the further increase in numbers of these bacteria (Fig. 10.6).

Initially salmonellas were inhibited by the litter presumably due to the action of the fumigant, this ability was soon lost so that the

*Presumptive counts, quantified on V-K Agar, organisms not identified further.

FIG. 10.6. THE PERISTANCE OF SALMONELLAS IN LITTER IN A COMMERCIAL ENTERPRISE SAMPLED WEEKLY.



salmonellas were able to multiply rapidly and then at about 30d the material became very inhibitory. In contrast in earlier experiments inhibition was detected as early as 17d showing that the rate of maturation of litters is very variable and must involve many parameters some of which may have not been considered in this study (Fig. 10.6).

These houses were managed by good commercial practice and so reflected accurately the condition prevailing in the industry and demonstrate the great complexity of this problem.

3. The Bacteria Flora of Litter from a Non-Commercial Poultry House Sampled at Weekly Intervals (Monitoring Exercise 10.3)

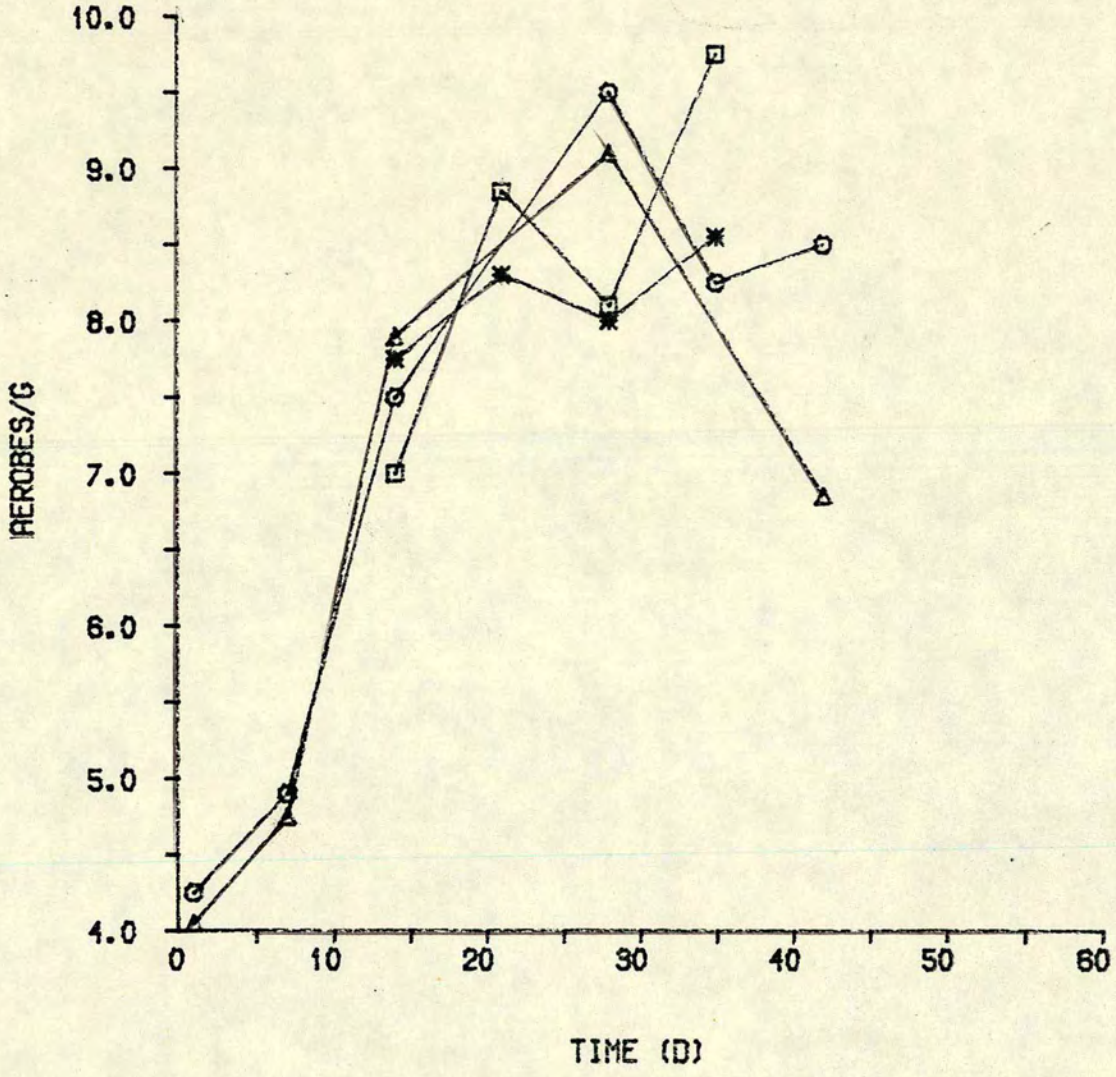
In a commercial poultry house there is a great deal of variation within the total area of the house. Normal commercial practice makes it impossible to ensure that all sampling stations are treated in the same manner. In experimental units the birds are kept in small pens which as far as practically possible are treated in an identical manner. The husbandry of these units is also of a higher standard than is often possible under commercial conditions. Advantage was taken of a trial being undertaken at the Poultry Research Centre (Roslin, Midlothian) where birds were being reared under identical conditions having been subjected to a variety of conditions during incubation and hatching.

Experimental Details

The poultry house contained 40 individual pens divided by 600mm high plywood boards with an upper portion of 500mm, 30cm square mesh wire. The house was fumigated by heating paraformaldehyde, two

FIG 10.7. THE AEROBIC BACTERIA IN LITTER FROM A NON-COMMERCIAL ENTERPRISE.

(KEY: ○ PENS 1-8, △ PENS 41-48, □ PENS 10-17, ✱ PENS 32-39)



days before the trial commenced.

Chicks were placed in the house on two occasions the first batch occupying pens 1-8 and 41-48 and the second batch 10-17 and 32-39. Therefore the level of fumigant was determined only for the pens occupied by the first batch of birds.

Samples of litter were collected from each pen by a member of staff of the Poultry Research Centre and received at the laboratory within 3h of sampling. The following analysis was carried out imediately the samples were received at the laboratory:-

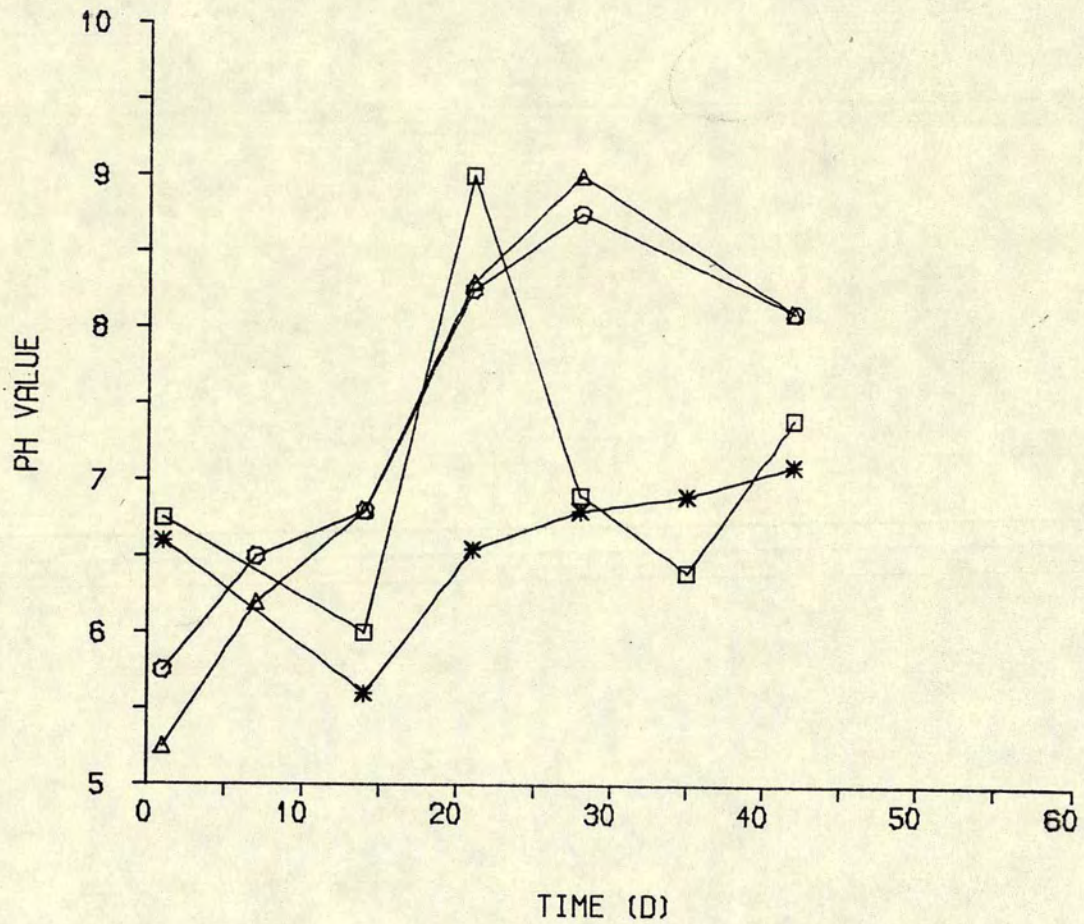
1. Total bacterial count on N agar, duplicate plates being incubated aerobically and anaerobically at 26°C.
2. The persistence of Salmonella typhimurium; 1g of litter was inoculated by 0.1ml of a 10^{-4} dilution of a overnight culture of S. typhimurium, incubated for 2d at 26°C after which the numbers of salmonellas were determined by the stated MPN-3 method.
3. pH value.
4. Moisture content.
5. Formaldehyde in new litter only.

Results and Discussion

The level of fumigant varied little between the pens although the right hand side of the house had a slightly higher level than the left hand side but the variation was insufficient to affect the development of the microflora of the litter. When the data pertaining to each pen was compared it was found that there was no

FIG.10.8. THE PH LEVEL IN LITTERS FROM A NON-COMMERCIAL ENTERPRISE.

(KEY: ○ PENS 1-8, △ PENS 41-48, □ PENS 10-17, ✱ PENS 32-39)



variation between the pens which could not be explained by experimental errors. Therefore the mean of the results of each group of 8 pens have been used in the following discussion of data.

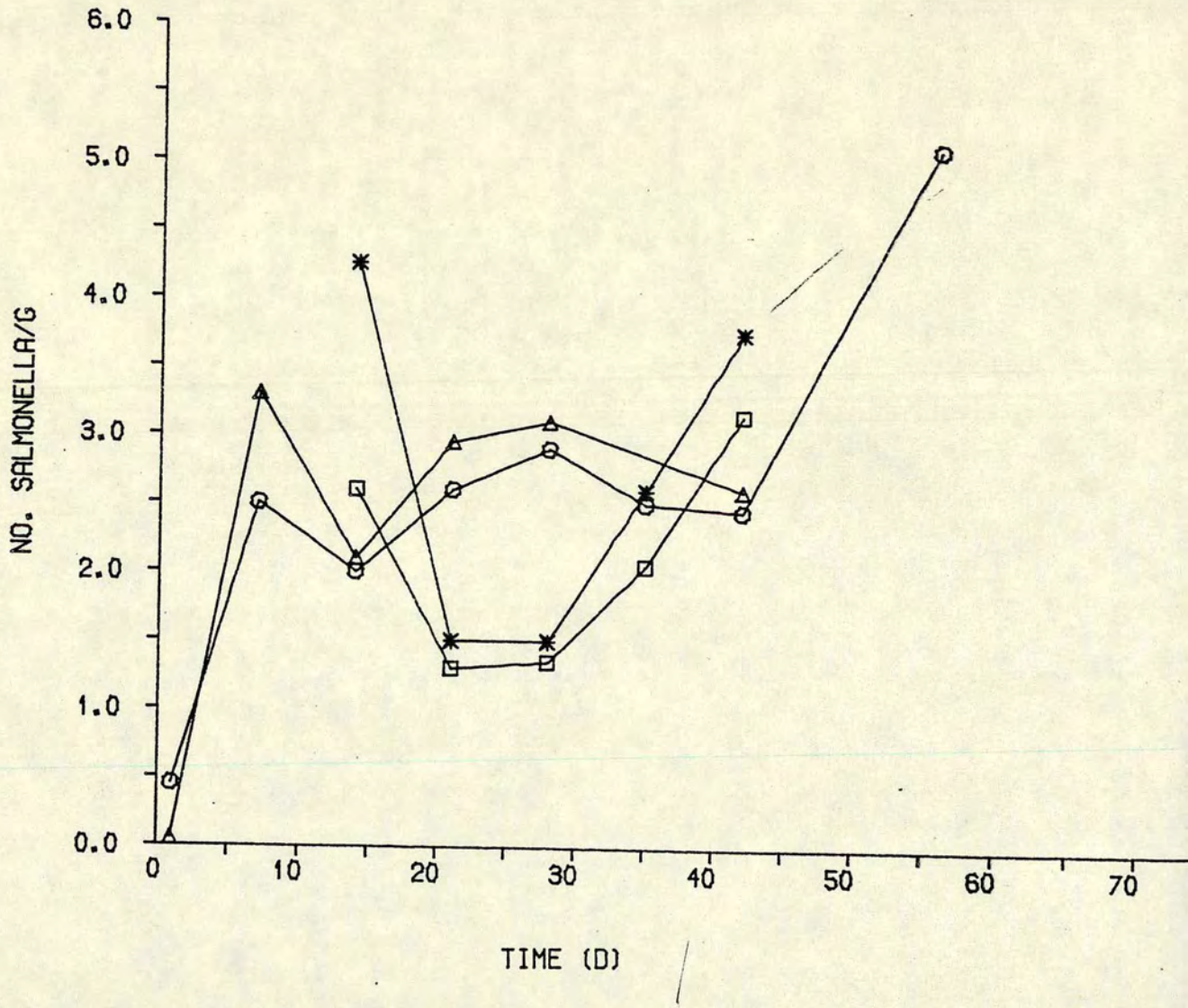
During the 42 days of the trial the aerobic count increased steadily from 5.0/g to 9.0-10.0/g (Fig.10.7). In contrast to the other series of litter examined there was no decrease in numbers after 30d. The moisture content rose from the initial level of 7.5-10.0% to 25.0-35.0% at 20d after which the moisture levels remains relatively constant in all pens. In contrast the pH levels varied between the groups of pens (Fig.10.8) the litters of the first batch of birds (pens 1-8 and 41-48) being less alkaline (pH 7-7.5) then the litters of the second batch (pens 10-17 and 32-39; pH 8-8.5). This variation did not appear to influence the numbers of bacteria in the litter although it may be speculated that the fumigant reduced the number of uric acid and urea decomposing bacteria in the first group of pens but there is no evidence to substantiate this.

None of these litters became inhibitory to salmonellas (Fig. 10.9) the litters from pens 10-17 and 32-39 becoming increasingly favourable to the growth of salmonellas while those from pens 1-7 and 41-47 permitted survival but did not allow multiplication.

Statistical analysis of these results showed no significant correlations exisitng between any of the parameters examined (Table A10 (iii)in Appendix 2). When those litters which favour the persistence of salmonellas are examined it can be seen that the salmonellas are found equally over a moisture range of 21-30% in pens 10-17 and

FIG.10.9. THE PERSISTENCE OF SALMONELLAS IN LITTER FROM NON-COMMERCIAL ENTERPRISE.

(KEY: ○ PENS 1-8, △ PENS 41-48, □ PENS 10-17, ✱ PENS 32-39)



32-39 while in pens 1-8 and 41-48 the moisture content was 25-35%. In addition the pH range for survival ranged from pH 7 to pH 8.

These results show that in the same house with small pens all the litters behaved in the same manner. However in this house the litters matured at much slower rates than would be the case in commercial houses.

D Discussion

The transformation of sawdust from a material with a low count of bacteria to an immature litter with a high number takes only five days however completion of the maturation takes a much longer period, the exact length of time varying from house to house. After initial variations the moisture content in all houses became relatively constant at 20-30% possibly affected by the rate of ventilation rather than changes within the litter per se. In contrast the pH value changes dramatically from a slightly acidic material to one with an alkaline pH value. The results of these experiments indicate that the changes in the litter result from the bacterial activity producing ammonia which is dissolved in the aqueous phase. The resultant change in pH level has a varying effect on the groups of bacteria, in that the total count of aerobic bacteria, Bacteriodes group, coli-aerogenes, stretococci, yeasts and moulds after initially increasing in numbers remain at a constant level. On the other hand the numbers of anaerobic bacteria, lactic acid bacteria, urea and uric acid decomposing bacteria decreased as the pH value increased.

In all the litter the persistence of salmonellas followed the same pattern as seen in Monitoring Exercise 10.1 in that when the

chicks were placed the litter was very inhibiting, within 7d this ability is lost and these bacteria can multiply in the material. At 20d in the first series of litter and 30d in the second series the litters becoming very inhibitory to salmonellas but this was not found in the third series. However the litter from Monitoring Exercise 10.3 which was from a non-commercial house did not have the visual appearance of a mature litter even at 56d. When the results of the litters which proved to be inhibitory to salmonellas were compared it was found that in Monitoring Exercise 10.1 the samples had a moisture content of 22-30% and pH 7.8-8.2, in Monitoring Exercise 10.2 24-31% moisture content and pH 8.2, the corresponding values for Monitoring Exercise 10.3 being 12.5-37% and pH 5.75-7.5

The samples examined in these three experiments are from selected houses which may not necessarily by typical of all poultry houses this is particularly true of the litters in Monitoring Exercise 10.3. But these results show that the variations between litters produced under different systems of management and indicates the difficulty of defining a mature litter. Statistical analysis of these results confirm the complexity of these interactions as no correlations were forthcoming when all the parameters were examined. This may be due to incorrect selection of parameters to elucidate this phenomenon or that the infinite number of variations involved in the interaction of bacterial and physico-chemical parameters defy resolution in the light of our current knowledge.

In this investigation no detailed study of the bacterial species isolated from the litter at each stage of maturation was undertaken

but the change of pH value from pH 5.6 to pH 7.5-8.5 indicates that there was a selection of bacteria able to utilise urea and uric acid to form ammonia and of those bacteria able to survive in these conditions.

In the following section these bacterial interactions will be further investigated in order to ascertain whether the change in pH value is the sole mechanism of inhibition of bacteria or if other inter-bacterial antagonisms are involved.

SUMMARY

1. The number of bacteria increase greatly in the first weeks and then except for lactic acid bacteria remained at a constant level until the end of the rearing period.

2. The pH value of the litters rose in the first 3w to pH 8 and remained at that level for the duration.

3. Initially formaldehyde in the litter resulted in inhibition of salmonellas but later salmonellas multiplied but after 20d in Exercise 1 and 30d in Exercise 2 the latter become inhibitory. This confirms the results of Survey 2.

4. In a non-commercial house the litter never become inhibitory.

5. Litter of birds kept in small pens did not replicate the conditions found in a normal commerical house.

CHAPTER XI

THE EFFECT OF MICROBIOLOGICAL FACTORS IN THE PERSISTANCE OF SALMONELLAS IN LITTER. 2. THE ACTIVITY OF ANTAGONISTIC BACTERIA.

THE EFFECT OF MICROBIOLOGICAL FACTORS IN THE PERSISTENCE OF SALMONELLAS IN LITTER. 2. THE ACTIVITY OF ANTAGONISTIC BACTERIA

A Introduction

The previous chapter has shown that litter contains a large number of bacteria from a wide range of genera. From this data no inference could be drawn as to which groups of bacteria are most likely to contribute to the inhibitory nature of mature litter. Therefore to distinguish the probable groups of bacteria responsible two approaches were followed, firstly enrichment techniques were used to select the desired group of bacteria and secondly conventional broth and agar culture techniques were explored to screen those bacteria able to grow on conventional media to detect those bacteria able to anatagonise salmonellas. Those cultures of bacteria able to inhibit salmonellas were then characterised and the possible mode of inhibition discussed.

1. Review of Literature

(a) The Bacteria of the Chicken Intestine

(i) The Microbes in the Gut of the Normal Chicken

The faeces of the chicken are the primary source of bacteria in the litter. Some genera may contribute to the fermentation process of the litter but not survive the ensuing changes and so not be represented in the microflora of the mature litter. Therefore it is worthwhile examining the literature pertaining to the gut flora of poultry in order to be able to anticipate the genera which may be present in the litter.

At the time the chick hatches the gut contents contain very few bacteria, numbers as low as 1.3-3.62/g being quoted by Shapiro & Sarles (1949) but even before feeding the number may rise to as high as 9.0/g with Streptococcus faecalis var. liquefaciens predominating according to Barnes et al. (1978). The numbers of these bacteria which are acquired from the environment probably varies greatly from hatchery to hatchery (Lev & Brigg, 1956a, Adler & Da Mossa, 1980). Once the chick has fed the number of "gut" bacteria always exceed the 9.0/g (Shapiro & Sarles, 1945; Lev & Briggs, 1958a and b; Mead & Adams, 1975), Clostridium spp. and Escherichia coli being found in addition to the faecal streptococci. During the following three weeks the constituent genera of the microflora change until a microflora typical of the mature birds becomes established (Barnes et al., 1972; Mead & Adams, 1974).

The earliest investigation of the bacteria of chicken gut was King (1905) who classified the dominant species as Bacillus coli-commis together with a few sporefomers, micrococci, streptococci and occasionally pseudomonads, sarcinea and yeasts. With improved isolation techniques Menes & Rochlin (1929) found Escherichia coli and Streptococcus faecalis in large numbers together with low numbers of Staphylococcus albus, S. citreus, Sarcina flavus, Bacillus subtitis, actinomycetes, yeasts and moulds. They also isolated Lactobacillus beijerinchi but in such low numbers that they could only be detected by an enrichment method. Emmel (1930) also found that Escherichia coli predominated with E. communis together with other aerobic and anaerobic bacteria. In contrast Johanson et al. (1948), Harrison &

Hansen (1950) and Rosenberg (1952) considered that the lactic acid bacteria were present in large numbers in the intestine but the latter authors also recovered larger numbers of anaerobic bacteria than other workers had up to that time. This predominance of anaerobes was confirmed by Ochi et al. (1964) who showed that lactobacillii and streptococci are the most numerous in the intestine with bacteriodes and bifidobacteria in the caecum. Using modern selective media these findings have been confirmed by Barnes and her co-workers at Norwich. During a thorough investigation by these workers they found that they were only able to isolate 20% of the 10.38-11.80 bacteria/g caecal material seen under the microscope (Barnes et al. 1972).

The intestinal organism most commonly isolated from the caecum by these workers are facultative anaerobes such as coli-aerogenes bacteria (especially Escherichia coli), Lactobacillus sp. and Streptococcus spp., non-sporing anaerobes including Bacteroides spp. Bifidobacterium spp., Coprococcus sp., Eubacterium spp., Fusobacterium spp., Gemminger sp. and other budding bacteria, Peptostreptococcus spp., Propionibacterium sp., Streptococcus spp. and spore-forming anaerobes of Clostridial group (Barnes et al., 1978).

During the last decade it has been established that the intestine and the caecum have distinctly different microflora (Barnes et al., 1972; Kimura, 1976; Salanto et al., 1978). However the now accepted view that the gut flora consists mainly of anaerobic bacteria has been questioned by Romoser et al. (1979)

who found equal numbers of aerobic and anaerobic bacteria in the samples which they examined.

(ii) The "Nurmi" Concept

In 1973, Nurmi & Rantala found that when an extract of mature chicken caecal material was administered to young chicks and then the chicks exposed to an inoculum of Salmonella infantis the chicks were found to have acquired "resistance" to these salmonellas. The following year it was found that the same method could be used to give resistance to S. infantis (Rantala & Nurmi, 1974). Since the publication of the method by the Finnish workers (Nurmi & Rantala, 1973) the effect has been confirmed in Australia (Lloyd et al., 1977), Canada (Ialzick & Cadwell, 1977; Rigby, 1977), Great Britain (Barnes et al., 1979; 1980a and b; Impey et al., 1982) and the United Stated (Snoeyenbos et al., 1978).

It has been shown that while turkey faeces (Lloyd et al., 1977) Snoeyenbos et al., 1978; Soerjadi, 1979) duck faeces (Soerjadi, 1979) and to a lesser extent the faeces of morning doves (Snoeyenbos et al., 1979) conferred such a resistance while bovine rumen liquor and horse-faeces did not have the same effect (Rantala & Nurmi, 1973; Soerjadi, 1979) indicating that the acquired factor is specific to avian species.

Rantala & Nurmi (1973) also showed that if the caecal material was cultured anaerobically in broth, the resultant mixed culture had the same attributes as the caecal material. However the bacterial species involved are probably not strict anaerobes as it is

possible to eliminate this factor by adding the culture to the drinking water (Impey et al., 1982) in feed (Rigby & Pettit, 1980; Impey et al., 1982) or by spraying into the air space of the hatchers at the hatcheries (Mulder pers. comm.).

Chicks treated in this way have also been shown to be protected from infection by other pathogens including Escherchia coli (Snoeyenbos et al., 1982; Wienach et al., 1981; 1982) Arizona hinschawii (Snoeyenbos et al., 1982) and Campylobacter jejuni (Soejadi et al., 1982) suggesting that the establishement of gut flora will exclude pathogens or more probably reduce their ability to multiply.

Increasingly this principle has become known as "competitive exclusion" but to date no evidence has been published to substantiate the assumption that this is the mechanism involved in the gut. A similar effect has been claimed for lactobacilli in humans (Tomic-Karovic et al., 1961), turkey (Francis et al., 1961) and chicken (Fuller, 1972; Fuller et al., 1979) protecting the animals from Escherichia coli (Wienach et al., 1982) but not Salmonella infantis (Adler & Da Massa, 1980). Investigation of these interactions has lead Soejadi et al. (1981b) to doubt whether this phenomenon is true competitive exclusion as they did not find evidence of receptor sites in the crop or caecum that are blocked by the introduction of the gut microflora. Hence instead of referring to this effect as "competitive exclusion" it would be preferable to use the term "antagonism" or the more ambiguous term the "Nurmi concept" which while giving credit to the originator of the method does not implicate any mechanism for this phenomena.

Considerable literature has now accumulated on this technique and the interaction of this technique and prophylaxis. While a comprehensive review would not be appropriate here some aspects are of significance in respect of the work reported in this thesis.

The important characteristics of this mode of inhibition are that:-

1. the site of action is within the chick gut
2. treatment makes them almost immediately resistant to salmonellas (Rantala, 1973)
3. it is possible to set up the phenomena by feeding chicks the intestinal contents of avian origin
4. or with a culture which has been incubated anaerobically (Rantala, 1974; Snoeyenbos et al., 1978; Rigby & Pettit, 1980)
5. these anaerobic bacteria are not fastidious (Rigby & Pettit, 1980)
6. the effect is negated by furazolidone (Rantala & Nurmi, 1974) and tetracycline (Rantala, 1974) when tested in laboratory media but not when bacitracin (Nurmi & Rantola, 1971) or nitrovin (Rantala, 1974) are the additive.

When added to feed nitrovin, bacitracin, furazolidone, gallimycin, penicillin/streptomycin, chlortetracycline and tylosin do not destroy the resistant effect (Nurmi & Rantala, 1971; Rantala, 1971; Snoeyenbos et al., 1979).

In Australia Soejadi et al. (1978) showed that Streptococcus faecalis isolated from the caecum has been able to induce the

effect in vitro and in vivo however the effect has not been confirmed by workers in Netherlands (Goren, 1981) and Canada (Piunick & Blanchfield, 19819. Barnes et al. (1979) had found that Bacteriodes hypermegas and a Bifidobacterium sp. were inhibitory in vitro but not in vivo. The latter two genera are included in the very effective "cocktail" of 48 organisms used by Impey & Mead (1981) and Impey et al. (1982). The Finnish workers (Schnietz et al., 1981) have attempted to classify the genera of bacteria in their mixture but although they were able to distinguish between 18 groups they failed to classify them to species level.

In the 1950's investigation had shown that chicks placed on old litter were less susceptible to infection than corresponding chicks reared on new litter (Anderson et al., 1953; King & White, 1953; Kennard et al., 1954). At that time the results were interpreted in terms of the nutrition of the birds, the reasoning being that the birds were deprived of minor feed additives, vitamin B_{12} being one factor which was positively identified (Halbook et al., 1950; Holbook et al., 1951; Kennard et al., 1954). A similar effect was achieved by King & White (1953) when they scattered chicken faeces on the feed in the troughs. In retrospect it would seem more probable that this effect was more likely to be due to the rapid acquisition of mature gut flora due to exposure to "dirty" conditions (Bare & Wiseman, 1961). This observation has recently been confirmed by Gustafsen (1983) who while investigating the effect of avoparcin to control the establishment of salmonellas in chicks found that chicks reared on old litter were less susceptible.

(b) Antagonism between Strains of Microbes

(i) Studies of Antagonism between Strains of Microbes

The possibility of antagonism was observed as early as 1874 by William Roberts who noted the inhibition of Penicillium glaucum by bacteria. Since that date a great deal of work has been carried out in particular in relation to plant and animal diseases and in soil microbiology. Among the literature reviewing this topic have been Noble & Pitcher (1977) on skin, Wohin (1974) and Savage (1977) on intestinal flora, Hardie & Marsh (1978) on dental plaque and Haltorri (1973), Bazin et al. (1976) and Stolzky (1972-3) on soil microbiology.

In nature microbes have become established in ecological niches in which they can compete and maintain themselves in association with other species (van Neil, 1955). While such a philosophy was first postulated in relation to the natural environment it also applied to man-made situations such as poultry litter.

Antagonism between micro-organisms was classified by Baker (1980) as:-

1. competition for nutrients, favourable sites and oxygen
2. antibiosis by metabolites of other bacteria
3. parasitism as predation of other micro-organisms.

As litter is formed by the mixing of bacteriologically active poultry faeces and biologically inactive sawdust or wood shavings the situation is dynamic in nature. However in a mature litter the physico-chemical conditons appear to remain stable so that a microbial community appears also to remain stable but in fact the microbial community which develops is an open system.

In an open system bacteria and nutrients are continuously being added in the form of faeces while there is constant leaching by drainage and evaporation, in such a system there are a wide range of microscopic ecological niches. For instance the spaces between the litter particles are indisputably aerobic, while anaerobic and micro-aerophylic conditions exist within the particles as has been shown for skin (Noble & Somerville, 1964) and root/soil interfaces (Rhodes-Roberts pers. comm.). Reactions such as the degradation of uric acid by bacteria from the chicken gut produces ammonia and carbon dioxide in the immediate vicinity which will render that micro-environment alkaline and micro-aerophilic. Each of the changes of conditions will favour some species of bacteria while being hostile to other strains.

There are many examples of antagonsims caused by bacterial activity which results in a modification of the environment, examples being the production of lactic acid (Tramer, 1966), acetic acid (Sornell & Speck, 1970) peroxide (Su, 1949; Wheater et al., 1952; Holmers & Hollander, 1973; Malke et al., 1974) volatile fatty acids (Walsted et al., 1974; Levison, 1973; Barnes et al., 1979). Such changes often lead to changes in pH or oxidation reduction potential (Kraft,1974; Ritz, 1967) which then result in a situation inhibitory to certain strains of bacteria. An example of this type of activity is the inhibition of Salmonella gallinarium by Leuconostoc citrovorium due to the production of acetic and lactic acids.

Microbes may also produce metabolites which can act directly

against the cells of susceptible strains. Such substances are variously termed antibiotics (Gratin, Fredericq, 1947), auto-inhibitors (Fredericq & Levine, 1967), microcins (Dalhoff, 1982) but as seen in a review on this subject by Tagg et al. (1976) there is a great deal of confusion in the terminology applied to these compounds.

The ability to antagonise bacteria is strain specific however the streptococci, pseudomonads and lactobacilli are particularly active in this respect.

While there are many reports of antagonism in the literature there are relatively few which involve salmonellas even although the inhibitory effect of some strains of coliforms to salmonellas and shigellas in broth was noted as early as 1916. More recently Flippen & Mickleson (1960) found four strains of Eschericia coli among 25 other genera of bacteria able to inhibit Salmonella senftenburg in egg albumin. They showed that in egg albumin at neutral pH the addition of E. coli reduced the numbers of salmonellas from 8.0/ml to 7.37/ml but the addition of 1% glucose resulted in a further reduction to 4.0/ml. Other species in their collection were Bacillus subtitis and B. polymyxa. The latter species had previously been shown to produce the antibiotic polymyxin which is active against salmonellas (Stansley et al., 1947). The activity of E. coli was similar in action, the agents being termed Colicines (Fredericq, 1957).

Not all the interactions between bacteria are detrimental to the bacteria concerned many consortia of bacteria used in the biotechnological industry being mixtures of strains which are able

to produce metabolites which are mutually useful (Wilkinson & Harrison, 1973; Lauke & Martin, 1981). In situations such as the oral cavity the models of the interactions include both antagonistic and stimulatory reactions the relationship being so complex that four methods of investigation were required to demonstrate this relationship (Halmbers & Hollander, 1971; Deyloff & Sanders, 1980).

(ii) Methods for detecting Interactions between bacteria

A great variety of methods have been published for the demonstration of interactions between bacteria these have included plate count (Hentges & Maier, 1970; Parker, 1970; Reddy et al., 1971; Maier & Hentges, 1972; Sevior & Board, 1972; Wilkinson & Harrison, 1973; Noble & Somerville, 1974) cross streaking aerobically (Fredericq & Levine, 1947; Holbert, 1948; Oblinger & Kraft, 1970; Carlson, 1971; Kolstat, 1975) and anaerobically (Kafel & Agrey, 1969; Barnes et al., 1979), seeding plate with stable culture Fredericq & Levine, 1947b), agar overlay (Deyloff & Sanders, 1980) reverse side streaking and direct lawnspotting (Holmberg & Hollander, 1972), inoculation of established cultures (Hentges & Maier, 1970) the Ecologen (Bidel et al., 1978), overlapping drops (Burrows, 1963), colicine method (Levine & Tanimolo, 1958; Burrows, 1963), stab with culture on plates (Coblentz & Levine, 1947) or in tubes (Burrows, 1973) by the stimulation of conditions (Parson & Miller, 1947) and the disc assay method (MacFarlane & Makriles, 1982).

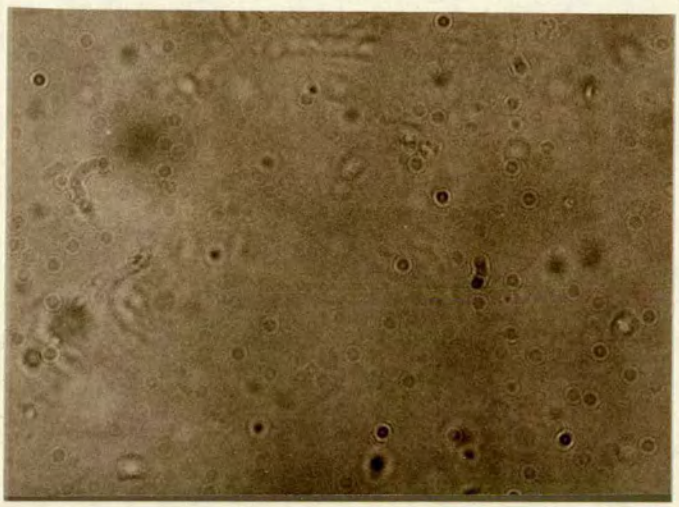
As there is a wide range of manners by which bacteria can

Photographs 11.1-3

The Persistance of Salmonellas in Agar Disc exposed in solution with varying dilutions to inhibit these bacteria.

Photograph 11.1

Total Inhibition

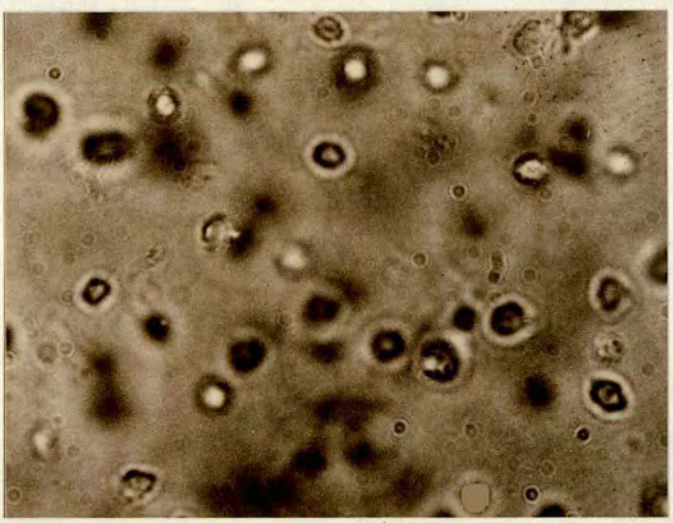


Photograph 11.2

Partial Inhibition

Photograph 11.3

Normal Growth



influence other strains it is often necessary to explore a number of techniques before demonstrating an antagonistic effect.

B Materials and Methods

1. The Dilute-to-Extinction Method for Isolating Pure Cultures or Simple mixtures of bacteria

Dilutions up to 10^{-12} decimal dilutions were prepared in Ringer's solution. 0.01ml of each dilution was added to 10ml of the appropriate culture media; 30 tubes being inoculated at each dilution. After incubation the solutions showing no visible growth were discarded. The selections from the two highest dilutions with some solution showing visible growth were retained as solutions possibly containing pure cultures or simple mixtures.

2. Detection of Inhibition by Agar Disc Method

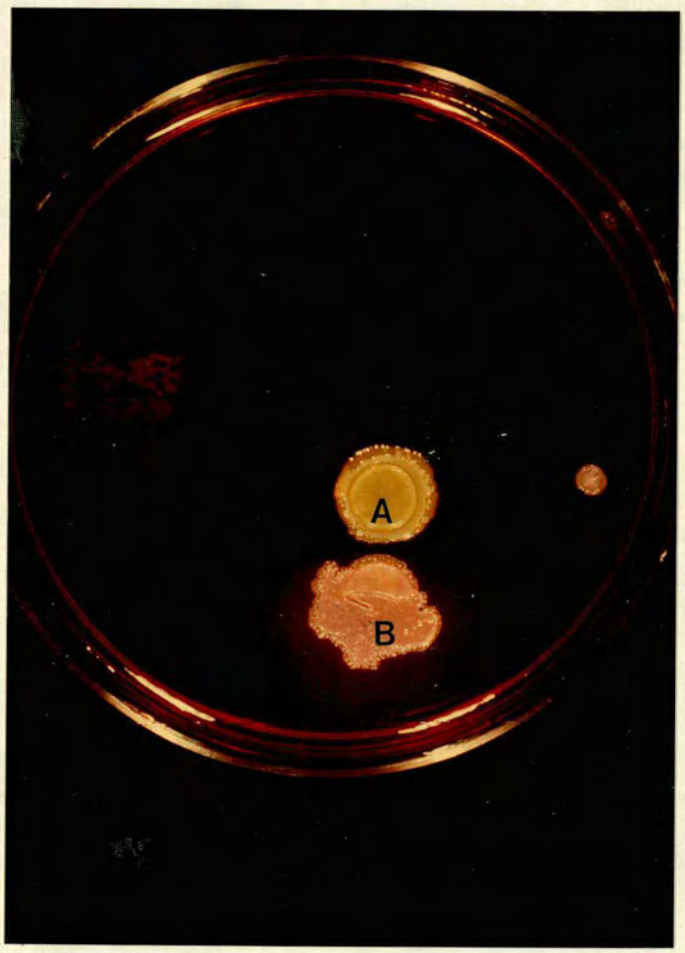
2.5ml of an overnight culture of Salmonella typhimurium was added to 250ml of sterile Water agar which had been rendered medium and cooled to 45°C. After gentle mixing 6ml of this seeded agar was dispensed into sterile petri dishes and allowed to set on a level surface. Using a cork borer of 7mm diameter discs of the seeded agar were removed and suspended in 2ml of the solution under test. 25 compartment dishes (Sterilin Ltd., Code No. 51021-103) were found to be convenient for this purpose although quarter ounce Bijou bottles were used on some occasions.

After incubation for the time stated the agar disc was removed from the solution using an inoculating loop bent at 70-90° and placed on a glass microscope slide. The growth of bacteria

Photograph 11.4

Resuscitation of Salmonellas in Water Agar discs after exposure in test solutions using Brilliant Green Agar as resuscitant.

[A-cells killed; B-cells inhibited but not physiologically damaged]



within the agar disc was examined by at x10 or x40 magnification and recorded as follows:-

I - Total Inhibition - no colonies (Photograph 1)

PI - Partial Inhibition - colonies small often disorganised or only a few large colonies present (Photograph 2)

\+ - Normal Growth - growth of colonies comparable to control discs (Photograph 3)

Control discs were prepared for each medium as the mode of growth varied slightly between each broth and group of discs.

Differentiation between bacteriostatic and bacteriocidal solutions was undertaken by transferring a replicate treated agar disc to 2-3ml of fresh N broth and incubating at 37°c for 24h. The agar disc was then examined and the degree of growth assessed according to the foregoing scale.

The extent of physiological damage to the cells was determined by comparing the growth of an agar disc incubated as above and one transferred to a dried plate as above and one transferred to a dried plate of BG agar. The plate being incubated at 37°C for 24h in an upright position. The bacterial growth from the agar disc (Photograph 4) was confirmed as salmonellas as described in Chapter 2 c (3).

Preliminary experiments showed that the appearance of the discs was identical whether incubation was at 26°C or 37°c therefore unless stated incubation was at 26°C for the initial monitoring stage and 37°C when the degree of inhibition to the salmonellas was assessed.

3. Preliminary Identification of Culture

(a) Morphology

The culture under consideration was inoculated into 10ml of N broth and the solution incubated at 30°C. At 12, 24, 36 and 48h a loopful of the solution was removed asceptically and placed on a clean glass microscope slide. After drying naturally at room temperature the smear was heat fixed and then stained with 1% aqueous solution of crystal violet (BDH Chemicals) for 5 min treated with gram's iodine (Appendix 1) for 1 min, decolourised with alcohol for less than 30 secs, counter stained with an alcoholic phenol carbol fuschin solution (Appendix 1) for 1 min and washed gently in tap water and air dried.

All stained smears were examined at a magnification of x100 under oil immersion using a Vickers microscope.

The morphology of these bacteria resembling coryneform bacteria were confirmed by the method of Curie & Keddie (1973). Six ml quantities of EYS agar was dispensed in a 9cm petri dish, which after solidifying on a level surface was dried open at 43°C for 15 min. 0.1ml of each culture was spread on the surface of one of the plates and the plates incubated at 22°C for 7d. At 6h, 12h, 24h, 3d and 7d a 1cm² section of the agar was removed and placed on a clean glass slide. A coverslip was then placed on the surface and representative field photographed under phase contrast illumination and an oil immersion lens.

(b) Colony Characteristics

The colony formation was determined on N agar for all strains.

(c) Catalase Test

A suspension of the culture was prepared by emulsifying a colony growing on N agar with a loopful of Ringer's solution on a clean glass microscope slide. One drop of "5 volume" hydrogen peroxide (BDH Chemicals Ltd.) was added to the suspension. The production of effervescence indicated a positive reaction.

(d) Motility

Motility was determined using the "hanging drop" technique (Collins & Lyne, 1976).

(e) Biochemical Tests

The API 20E system (API SA France) was used as an aid for the identification of the pure cultures. The following reactions were carried out on each gallery, the production of o-nitrophenyl galactosidase, reduction of nitrate to nitrate, proteolysis of gelatine, hydrolysis of urea, indole production from tryptophan, acetoin from pyruvic acid, utilisation of citrate, presence of cytochrome oxidase, hydrogen sulphite production and the fermentation of saccharose, L (+) arabinose, mannitol, fructose, glucose, maltose, rhamnose, galactose, mannose, sobitol and glycerol.

(f) Oxidation-Fermentation of Glucose

Five ml quantities of oxidation-fermentation medium (O/F media) (Hugh & Leifson, 1953) were heated to 100°C for 5 mins and immediately cooled to 43°C when 0.1ml of 10% sterile glucose solution was added and mixed into the medium by rolling the tubes by hand. When

the medium had cooled to room temperature it was inoculated by stabbing a small quantity of the bacteria in question into the solid agar. Duplicate tubes were prepared for each culture, one tube being incubated aerobically and the remainder under anaerobic conditions.

After 48h incubation at 26°C the agars were examined for the colour change of green to yellow indicating the fermentation of glucose.

(g) Aerobic Decompositon of Uric Acid

A loopful of a 3d culture of appropriate bacteria in N broth was streaked on a previously dried plate of uric acid decomposing agar (Stapp, 1920). The plates were incubated at 26°C for 7d. Clearing of the opaque layer of uric acid was recorded as a positive results.

(h) Hydrolysis of Hippurate

Ten ml quantities of sodium hippurate broth (Cowan & Steel, 1974) were inoculated with 1 loopful of a 3d culture of bacteria in question grown in N broth. The solution was incubated at 30°C for 7d. A brownish-pink precipitate soluble in excess 5% ferric chloride aqueous solution (BDH Chemicals Ltd.) was recorded as a positive result.

(i) Litmus Milk

Ten ml quantities of Litmus Milk were inoculated with one loopful of a 3d culture of the bacteria under test and after mixing incubated at 26°C for up to 5d when the results were recorded

according to the following code:-

NC - no change; R - reduction (litmus decolourised); A - acid (litmus slightly pink or pink); AC - acid clot; (Litmus pink and milk clots); AKI - alkaline (litmus slightly blue or blue); SC - sweet clot (litmus unchanged or blue clot soft easily broken); D - digested; G - gas production.

(j) Production of acidity from glucose

A loopful of the appropriate bacteria was spread on the surface of a previously dried plate of glucose agar and incubated at 22°C for 10d. The rate of change of colour of the indicator in the vicinity of the colonies from purple to yellow was recorded.

(k) Diastase Production

A loopful of a 3d culture of the bacteria under test was streaked on a previously dried plate of peptone yeast extract agar containing 1% soluble starch and the agars incubated for 10d at 22°C. After incubation the surface of the plate was flooded with 1% iodine solution. A clear zone around a colony was recorded as positive for the presence of the enzyme diastase.

(l) Liquifaction of Gelatine

The ability of the bacterial strain to liquify gelatine was determined by inoculating, by stabbing, a colony of the strain grown for 3d on N agar into 10ml quantities of Nutrient Gelatine Agar in 16mm test tubes which were then incubated at 32°C for 10d.

The tubes were examined for liquifaction at 3, 6 and 10d after refrigeration for 30 min prior to recording.

(m) Haemolysis

A loopful of the culture to be tested was streaked on a previously dried plate of DST agar containing 7% (v/v) horse blood (Oxoid Ltd.) and incubated aerobically for 3d at 30°C. The haemolytic reactions was recorded immediately and after incubation at room temperature in the light for 3d. The reaction was recorded as follows:-

α -haemolysis - narrow green band; β -haemolysis - clear zone surrounding colony; γ -haemolysis - no visible effect.

(n) Growth under anaerobic conditions

A loopful of a 3d culture of the appropriate bacteria was streaked on the surface of N agar which had been stored for 2d under anaerobic conditions and immediately replaced under anaerobic conditions. After incubation for 5d at 26°C the plates were examined for the presence of growth.

4. The Antibiotyping of Bacteria Cultures

The pattern of antibiotic susceptibility of the culture was determined using Mastring-S system (Mast Laboratory Ltd.). 0.1ml of a 10^{-1} dilution of a 3d culture of the bacteria to be tested in N broth was spread over the surface of a dried plate of DST agar and allowed to adsorb onto the surface of the agar by standing on the laboratory bench for 30 min in the

vicinity of a lighted bunsen flame. "Mastring-S" were applied aseptically to the inoculated surface of the agar care being taken to ensure that the whole ring was in contact with the agar surface.

Three combinations of antibiotics were selected to enable each culture to be subject to the following antibiotics:-

ampicillin (25mg), chloramphenicol (50mg), colistin sulphate (100mg), kanamycin (30mg), nalidixic acid (30mg), nitrofurantoin (50mg), streptomycin sulphate (25mg), tetracycline (100mg), bacitracin (8mg), neomycin (30mg), penicillin (4mg), polymyxin (250mg), caphaloridine (25mg), sulphanethiozole (200mg) and cotrimaxozole (25mg).

After incubation at 30°c for 24h the plates were examined for zones of inhibition of at least 6mm in diameter which are recorded as positive for susceptibility to that antibiotic.

5. The Identification of Possible Inhibitory Substances

(a) Antibiotic Activity

Ten ml quantities of DST agar were seeded with 0.1ml of 10^{-1} dilution of an overnight culture of Salmonella typhimurium. These plates were dried open at 43°C for 1h. The test solution of culture was adsorbed onto a disc of filter paper (Whatman AA 6mm disc) the excess solution being removed by touching the surface of the dry neck of the culture vessel. The disc is placed on a predetermined spot on the seeded agar plate. After incubation at the stated temperature for 24h the agars were examined for the presence of

a clear zone of at least 6mm around each disc which is recorded as a positive reaction.

(b) Identification of Possible Inhibitory Substances by Paper Chromatography

A 25cm square sheet of Whatman No. 1 chromatography paper was taken. A pen line was drawn 3cm from one edge and beginning 3cm from one end a pencil dot marked and further dots indicated at 2cm intervals. Each dot represented an origin for each solution under test, the code for each solution being indicated below these markers.

Using a finely drawn Pasteur pipette 5 drops of each solution was applied to the appropriate origin, marked on the paper, time being allowed for each drop to dry before the application of the next drop. Drying was accelerated by placing the sheet on a heated surface. The appropriate reference chemicals were included on each chromograph. When all the origins had been inoculated and dried, the sheet was formed into a cylinder the edges being fastened together using tongued clips or stainless steel paper clips. Care was taken to ensure that the edges did not overlap.

Approximately 50ml of the appropriate solvent was placed at the base of a chromatography tank (Shandon Unikit), the lid replaced and the tank allowed to stand overnight to ensure that the atmosphere was saturated with the solvent.

The prepared paper cylinder was lowered into the tank the inoculated end lowermost ensuring that it was freestanding within

the tank. The lid was replaced and the assembly allowed to stand for the appropriate time. The paper cylinder was then removed and the solvent front marked with a pencil mark. The sheets were dried by hanging in a draught in a fume cupboard.

The separated substances were located by spraying the dried chromatogram with the appropriate reagents. After drying a permanent record was made by drawing a pencil outline around the coloured spots.

C Experimental Work

(a) Selection of Bacteria from Poultry Litter able to inhibit Salmonellas

Poultry litter has a microflora derived from microflora of the feedstuff after selective action of the digestive tract of the chicken. Therefore it could be argued that the bacterial species involved in the inhibition of salmonellas in litter are equally likely to be recovered from poultry feedstuffs or chicken faeces.

(i) The Use of a Chemostat to Establish an Inhibitory Mixture of Bacterial Species (Experiment 11.1)

Chemostats are very useful in establishing mixed cultures which can be maintained in a stable condition for a considerable length of time (Brown et al., 1978). If such a mixed culture developed in a chemostat was inhibitory to salmonellas it would be possible to

ensure continuity of the inoculum while the mode of inhibition was being investigated. Therefore this technique was explored to assess the feasibility of this method in the context of this investigation.

Experimental Details

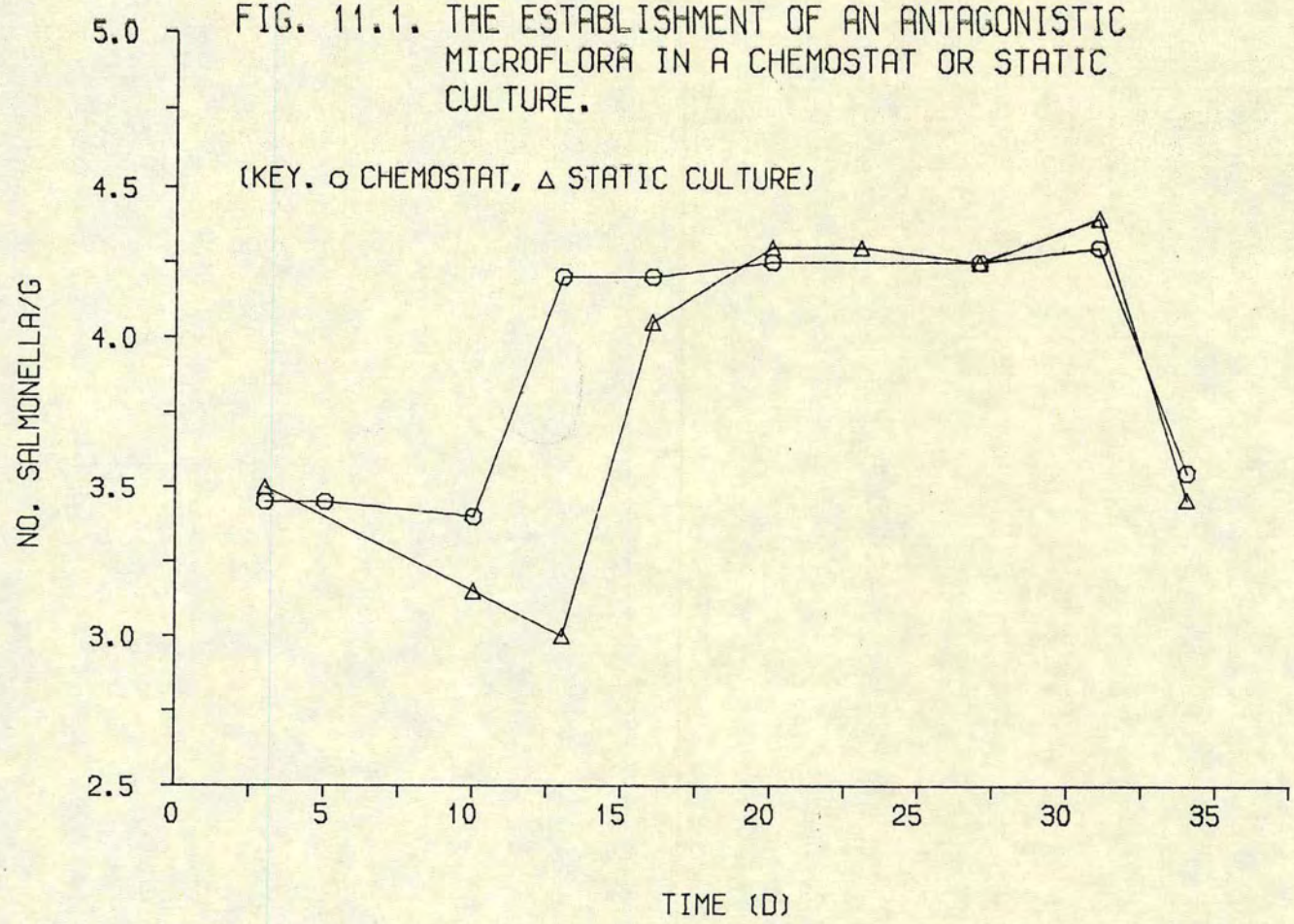
A New Brunswick C-30 bench-top fermenter with a working volume of 305ml was set up. Two hundred ml of N broth was inoculated with 10ml of 10% extract of 5d litter (L 43). The culture was maintained statically for 2d after which dilution was initiated with sterile N broth at the rate of 1.2-1.8l/min, the dilution rate being controlled by a peristatic pump (Pharmacia). The vessel was aerated at 78 culture volumes L^{-1} while maintained at room temperature (16-20°C). Samples were taken directly from the culture vessel using a hooded sampler discarding the first sample which contained material from the sampling line dead space.

At intervals of 2d a 10ml sample of the broth culture was challenged with Salmonella typhimurium by inoculating 0.1ml of 10^{-3} of an overnight culture of the salmonella. After incubation for 2d at 26°C the number of salmonellas were enumerated using the stated MPN-3 method. The experiment was terminated at 4w.

A static cutlure (I_3) was maintained in a 2l conical flask at room temperature.

A second culture derived from the faeces of a 4w hen was used in the next run. However even at the lowest flow rate (0.2d/min) the cultured "washed out". The corresponding static culture grew

FIG. 11.1. THE ESTABLISHMENT OF AN ANTAGONISTIC MICROFLORA IN A CHEMOSTAT OR STATIC CULTURE.



successfully and so this culture was used to replace the broth in the fermentation vessel. This culture was not maintained in the fermentation vessel even when aeration was ceased for 7d.

Results and Discussion

The culture selected from the litter inoculum did not inhibit salmonellas (Fig. 11.1) and so was discarded. The corresponding static culture was found to be inhibitory at 4w and so was returned for further investigation.

It was not possible to maintain a culture derived from faeces in the fermentation vessel with or without aeration and a very slow dilution rate. As the static culture grew well this would seem to indicate that these bacteria were favoured by anaerobic or micro-aerophilic conditions and could not establish in the aerobic condition of the chemostat.

The results of this experiment were not sufficiently promising to justify modification of the chemostat in order to maintain anaerobic conditions and so this technique was abandoned.

(ii) The Enrichment of Bacteria from Poultry Feedstuffs and Faeces in Static Culture (Experiment 11.2)

The previous experiment showed that mixed cultures inhibitory to salmonellas could be derived using static cultures. Therefore an enrichment technique included with poultry feedstuffs and faeces in static cultures was investigated.

Table 11.1

The Persistence of Salmonella typhimurium in solution enriched by a mixture of bacteria derived from Poultry feed and faeces (figures in parenthesis are pH value at 7d. Raw data in Table A 11 (i) in Appendix 2)

Initial pH	% urea	Number of salmonellas/ml at 7d in solution inoculated with Mash	Pellets	Faeces
4.5	0	6.37 (8.7)	6.38	2.36(8.9)
4.5	1.0	1.36 (9.1)	1.36	0.36(9.1)
7.0	0	5.38 (8.9)	3.36	3.38(8.8)
7.0	1.0	1.36 (9.1)	1.36	0.6 (9.0)
8.5	0	4.63 (9.0)	4.63	1.66(9.0)
8.5	1.0	1.63 (9.2)	1.63	0.95(9.1)

Experimental Details

Samples of chick mash (A) and rearing pellets (B) were collected from troughs in poultry houses. Faecal samples were obtained from laying hens at Easter Howgate Poultry Unit. 0.5ml of each material was added to 50ml quantities of N broth the pH of which had been adjusted to pH 4.5, 7.0 and 8.5. Duplicate broths containing 1% urea were also inoculated in a similar manner. The solutions were inoculated at 26°C.

At 2d, 7d and 10d, 10ml of each solution was removed and incoulated by 0.1ml of a 10^{-2} dilution of an overnight culture of Salmonella typhimurium and these inoculated solutions incubated for a further 2d at 26°C. The number of salmonellas were then determined by the stated MPN-3 method.

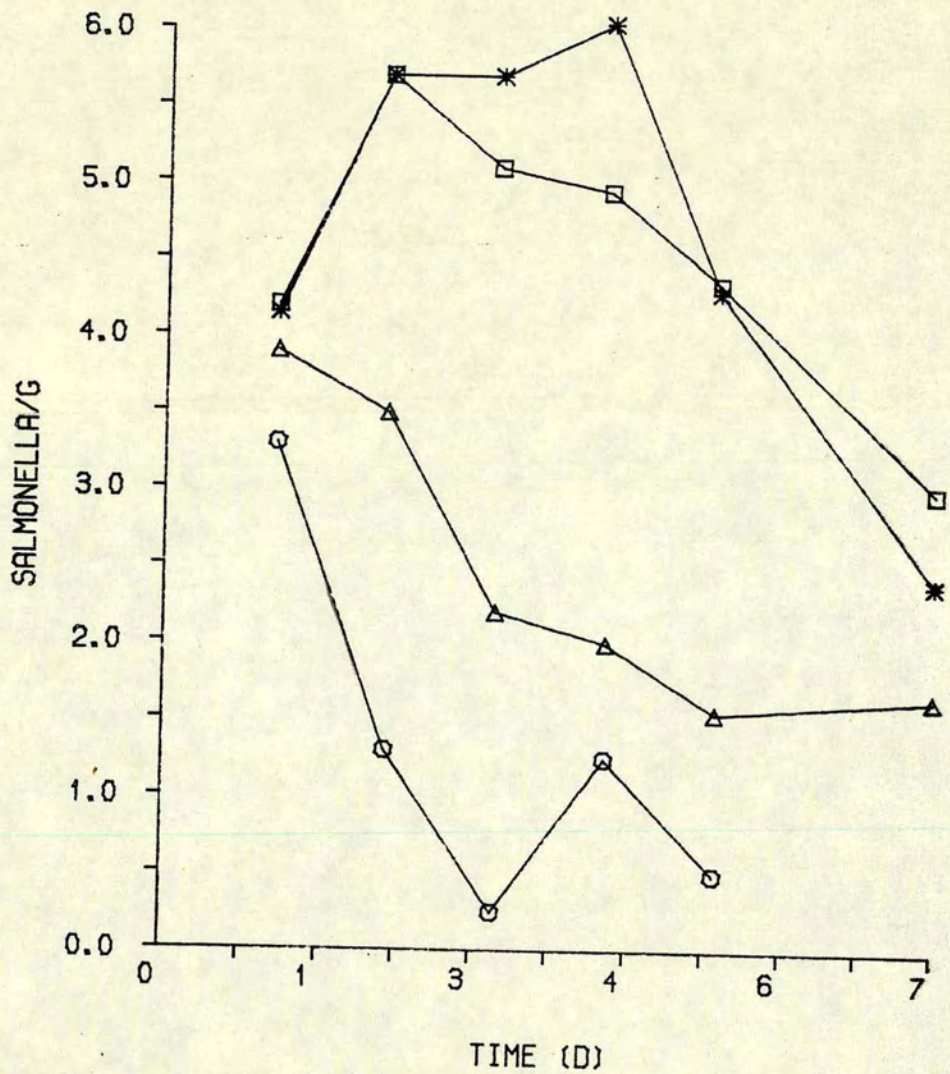
The solution derived from the feed pellets (B) was incubated for a further 14d.

Results and Discussion

None of the enrichment broths were able to completely prevent the growth of salmonellas (Table 11.1). The greatest inhibition in the solutions containing urea was probably the result of an increase in pH rather than direct bacterial action. In this experiment the conditions appeared to favour those bacteria able to decompose urea to ammonia which in turn lead to an increase in alkalinity and so reducing the survival of salmonellas. These results show that in alkaline conditions of over pH 8.5 a change of pH of 0.1 unit can result in a reduction of growth of over 1 $\log_{10}$

FIG. 11.2. THE SELECTION OF AN ANTAGONISTIC FLORA FROM THE BACTERIA IN MATERIALS FROM THE POULTRY INDUSTRY.

(KEY: ○ POULTRY FAECES, △ OLD LITTER, ✳ PELLETED FEED, □ NEW LITTER)



Hence it would appear that the growth of salmonellas are very easily inhibited either by a slight increase in alkalinity or by another inhibitory agent.

(iii) The Role of Ammonia in the Inhibition of Salmonella typhimurium by the Modified Microflora (Experiment 11.3)

The previous experiment indicated that the presence of ammonia rathe than direct antagonism between the indigenous bacteria was inhibitory the salmonellas. The following experiment was carried out to examine the effect of ammonia on the persistence of salmonellas.

Experimental Details

The pH of N broth was adjusted to pH 4.2, autoclaved at 121°C for 15 min, and a Seitz filtered solution of 40% urea in N broth added to give a final concentration of urea of 16%. Volumes of 400ml of the broth were placed in 500ml glass bottles and the following added to individual solutions:-

1. 4g pelleted poultry feed
2. 4g faeces from mature laying hens from the Easter Howgate Poultry Unit
3. 4ml of 10% raw extract of 3d litter
4. 4ml of 10% raw extract of 42d litter

The bottles were incubated at 26°C and at 24h intervals, 10ml quantities of the solutions were removed to be challenged with S. typhimurium as described in Experiment 9.2. The ammonia present was assessed accurately by the method currently in use at the Nutrition Chemistry Department of the East of Scotland College of Agriculture.

Table 11.2

Summary of Statistical Analysis of the Interaction of Parameters in Enrichment Broths Containing Four Materials

Parameters tested	Regression Coefficient (R^2) for:- Feedstuff	Hen Faeces	3d litter	6w litter
Day v pH	88.5	71.7	85.0	59.6
Day v NH_4	96.0	78.3	98.0	9.0
Day v Salmonella	36.1	13.2	32.2	93.4
pH v NH_4	89.7	61.7	63.3	13.6
pH v Salmonella	46.6	35.7	20.8	68.6
NH_4 v Salomella	59.9	97.7	99.3	0.0

Results and Discussion

In the 7d duration of the experiment the conditions in the solutions derived from the faeces and the mature litter become hostile to salmonellas. On the other hand the addition of feed pellets and 3d litter although they reduced the numbers of salmonellas slightly could not be considered as inhibitory (Fig. 11.2)

When the correlation coefficient of these inter-relationships was calculated it could be seen (Table 11.2) that in all the solutions except for those solutions containing the 6w litter inhibition of the salmonellas was related to the increased alkalinity resulting from the accumulation of ammonia. Therefore the bacteria selected during these enrichment periods are able to degrade urea to ammonia which then by virtue of the change of pH of the solutions was able to inhibit salmonellas. The solution fromthe 6w litter appears to have a different mode of inhibition for although the solution became more alkaline this increase in pH was completed in the first 2d and does not correlate with the ammonia level. This discrepancy suggests that there is an additional factor exerting an effect in this particular solution.

(iv) Modification of the Microflora derived from Poultry Feedstuff and Faeces by the Addition of Antibiotics (Experiment 11.4)

Workers investigating the phenomena of competitive exclusion in chicks have demonstrated that the inhibitory effect of cultures used to inoculate chicks could be negated by the addition of

Table 11.3

The Effect of the Addition of Antibiotics to Cultures derived from Poultry Materials

Antibiotics	Number of Salmonellas persisting in:- Original Broth	After Transfer
Streptomycin sulphate	<1.00	<1.00
Oxytetracycline	7.38	5.38
Furazolidone	5.38	6.32

streptomycin sulphate and oxytetracycline but not furazolidone. Therefore the possibility of using these findings to isolate potentially inhibitory bacteria was examined.

Experimental Details

Streptomycin sulphate, oxytetracycline and furazolidone were added individually at 0.001% level to 100ml quantities of N broth. One ml quantities of the enrichment broth from Experiment 11.3 was added to each solution and these solutions incubated at 26°C. After 5d incubation 10ml of each solution was removed, inoculated with 0.1ml of a 10^{-2} dilution of an overnight culture of Salmonella typhimurium and further incubated at 26°C for 2d when the number of salmonellas was assessed by the stated MPN-3 method.

Additionally at 5d, 1ml of each solution was added to separate 100ml quantities of a sterile N broth and these solutions incubated for a further 5d when it was inoculated in the manner already described.

Results and Discussion

The addition of oxytetracycline and furazolidene eliminated a proportion of the inhibitory effect of the solution to S. typhimurium but this did not occur when streptomycin sulphate was added (Table 11.3). The agreement with the results of the inhibition of the original broths containing a relatively high concentration of antibiotic and the solution gained by transfer of the original to a fresh solution which would then contain a low level of antibiotic shows that the inhibitory effect is due to bacterial action rather than the effect of the presence of the antibiotic.

These results contrast with those found when these antibiotics are given as a prophylactic to chicks in conjunction with treatment with culture according to the "Nurmi concept". Therefore it would appear to indicate that the same bacterial species are not involved in both phenomena. As streptomycin sulphate at 50µg/ml concentration is active against 87% of strains of lactobacilli, 50% of Escherichia coli and 32% of streptococci when tested by Kitai & Arakowa (1978) it is possible that these species of bacteria are not involved in this inhibitory action.

The resistance of the bacteria concerned with the in vitro inhibition of salmonellas by streptomycin sulphate could be an attribute which may be used to recognise those bacteria which are involved in the mechanism of inhibition of salmonellas in poultry litter.

(b) The Isolation of Bacteria responsible for Inhibiting Salmonella typhimurium in Litter from Enrichment Solutions

The dilute-to-extinction method and conventional plating techniques were investigated to select those bacteria in the broths which are responsible for the inhibition of salmonellas.

(i) Isolation of Bacteria responsible for the Inhibition of Salmonella typhimurium in Enrichment broths by Dilute-to Extinction Method (Experiment 11.5)

Experimental Details

Decimal dilutions up to 10^{-12} were prepared from the solution in Experiment 11.3 after 7d incubation. Using the dilute-to extinction

method pure cultures and simple mixtures of bacteria were obtained after incubation at 26°C for 2d. These solutions were then inoculated with 0.02ml of 10^{-2} dilution of an overnight culture of Salmonella typhimurium which were incubated for a further 2d at 26°C. One ml of each solution was then added to 10ml LINCR broth and these solutions incubation at 37°C for 2d to determine the presence of salmonellas. Those LICNR broth showing the colour change indicating a positive reaction to salmonellas were confirmed by streaking one loopful of the solution on previously dried BG agar plates and these incubated at 37°C for 24h.

The solutions derived from the 10^{-8} and 10^{-9} dilutions were incubated for a further 5d at 26°C when they were re-examined in the manner described above.

The presence of ammonia was determined in all tubes not showing the growth of salmonellas using Nessler's solution.

Results and Discussion

After 2d salmonellas were able to grow in all the solutions tested. After 7d incubation 20 of the 724 original solutions which showed turbidity were able to inhibit salmonellas. This constituted 2.77% of the strains or simple mixtures of bacteria from the original dilutions. All these solutions had a pH of over 8.5 and a high concentration of ammonia. It can therefore be concluded that the inhibition was due to the alkalinity caused by ammonia.

(c) The Antagonism of Bacteria to Salmonellas without modification of environment

While the change in pH level resulting from production of ammonia appears to be one mechanism of inhibiting salmonellas in litter it is possible that other mechanisms may be active possibly by direct antagonism of bacteria of the indigenous microflora to the salmonellas.

(i) The Detection of Antagonism of Bacteria to Salmonellas by Conventional Plating Techniques (Experiment 11.6)

Experimental Details

The solutions from Experiment 11.3 were plated in duplicate on N agar. One set of plates was incubated aerobically and the other anaerobically, incubation on both occasions being at 26°C for 2d.

Those plates with discrete colonies were selected and the agars removed intact and reversed into a sterile petri dish. Ten ml of N agar seeded with 0.1ml of a 10^{-2} dilution of an overnight culture of Salmonella typhimurium was used to seed the inverted agar. The plates were incubated at 37°C for 24h and then examined for zones of inhibition in the layer containing salmonellas.

Results

No zones of inhibition were noted.

(ii) Examination of Solutions demonstrating Inhibition to Salmonellas by Conventional Plate Methods (Experiment 11.7)

The mode of inhibition of the cultures isolated in the previous experiment which had been inhibitory to salmonellas in both cultures were further investigated using a wide range of conventional plate methods.

Experimental Details

The 20 solutions containing pure cultures of simple mixtures of bacterial species in Experiment 11.5 that were able to inhibit salmonellas were examined by the following methods.

(a) Disc Assay

A previously dried plate of DST agar was taken and 0.1ml of 10^{-2} dilution of an overnight culture of Salmonella typhimurium was spread on the surface and the plate dried for 1h at 37°C. Disc of filter paper (Whatman AA discs 6mm) were dipped into the solution under investigation, excess liquid drained by touching the side of the containers and then transferred to a predetermined point on the inoculated plate. The plates were incubated upright for 24h at 37°C when they were examined for the presence of zones of inhibition.

(b) One ml of each solution was added to sterile quantities of N agar and DST agar which had been rendered molten and cooled to 45°C and after mixing each poured into a sterile petri dish and allowed to solidify. 0.5ml of a 10^{-2} dilution of

an overnight culture of S. typhimurium was spread on the surface of the inoculated agar. The plates were incubated at 26°C for 2d when they were examined for the growth of salmonellas.

(c) The above method was repeated except that the inoculated plates were incubated for 5d at 26°C before the surface was inoculated with salmonellas.

(d)-(e) The methods described in (a) and (b) were repeated except that the surfaces of the inoculated agar were overlaid with the appropriate agar after seeding with S. typhimurium.

(f) Previously dried plates of N agar and DST agar were marked into 6 segments per plate. One loopful of each solution was streaked in each segment and the plates incubated at 26°C for 5d. Five ml of the appropriate agar was melted and cooled to 45°C and 0.05ml of an overnight culture of S. typhimurium added, mixed and poured over the surface of the cultured agar. After incubation at 37°C for 24h the plates were examined for the presence of zones of inhibition.

(g) The above method (d) was repeated except that each inoculated agar layer was reversed into a sterile petri dish before the seeded layer was added.

(h) The above method (d) was repeated except that the surface of the agar plate was sealed with the appropriate agar prior to the initial incubation.

Results No inhibitory activity demonstrated

3. The Investigation of Inhibition of Salmonellas by Bacteria from the Indigenous Bacteria using the Agar Disc Method

Antagonism of salmonellas by bacteria from the indigenous bacteria

was not shown by the conventional techniques although it could be demonstrated in broth culture. In 1982 a method for detecting interactions between bacteria using a seeded agar disc was published (MacFarlane & Makriles, 1982). The technique involves suspending a seeded agar disc in a broth culture or sterile broth and noting the manner of growth of colonies within the disc. Preliminary trials suggested that this method could be useful in detecting antagonistic activity in the context of the inhibition of salmonellas in poultry litter.

As the dilute-to-extinction method had proved unsatisfactory for the isolation of antagonistic bacteria therefore a plating technique was used to isolate potentially inhibiting strains of bacteria in the following experiments. Replicate plates were incubated in three gaseous environments to isolate as wide a range of bacteria as possible.

(a) The Isolation of Antagonistic Bacteria from Poultry Litter

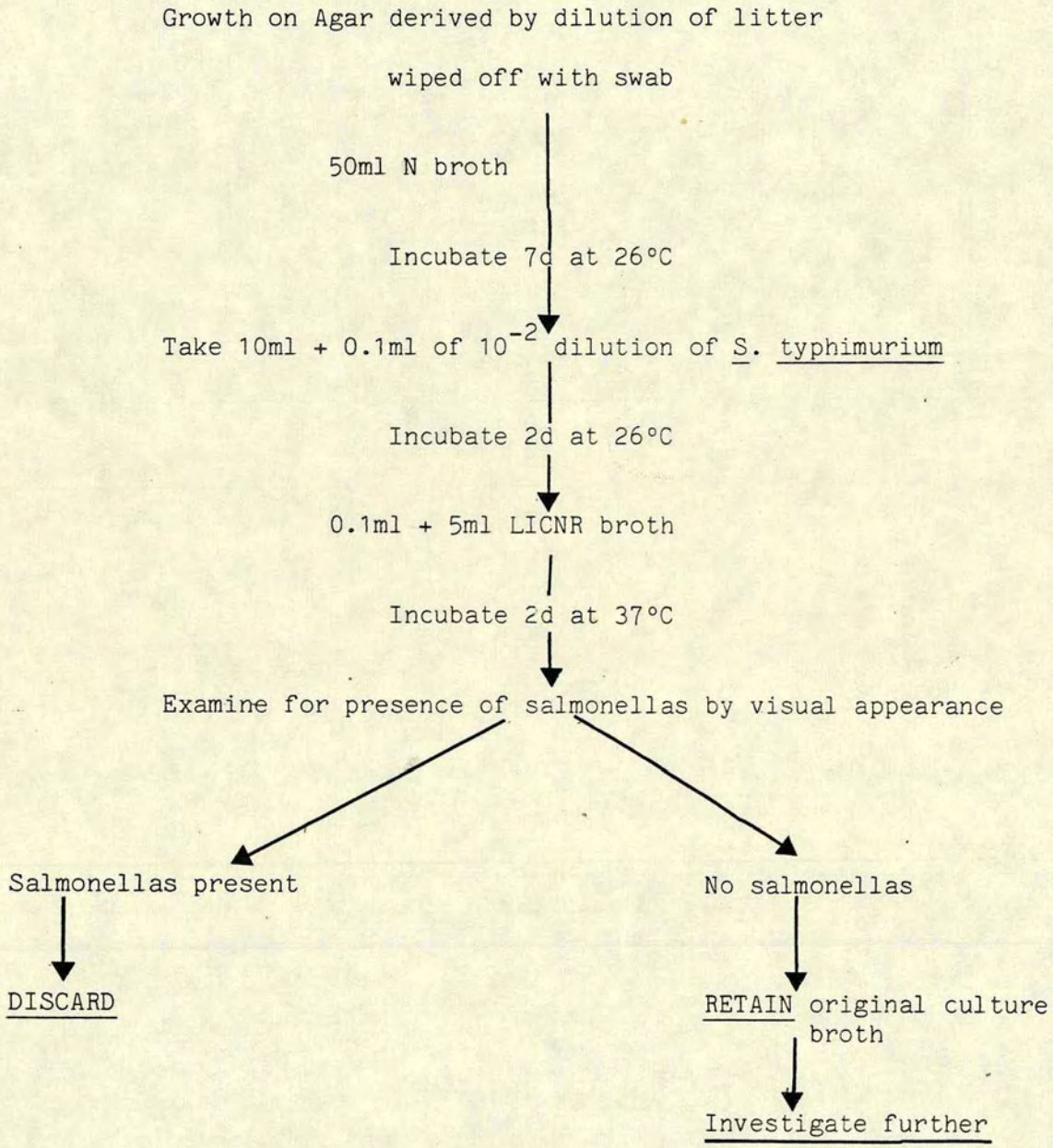
Initial Screening (Experiment 11.8)

Experimental Details

Fifteen poultry litters were collected (L 44-58) from poultry units and within 6h of collection three replicate agar plates were prepared using 0.1ml of a range of decimal dilutions from 10^{-4} to 10^{-7} to inoculate the surface of the dried plate of N agar. One set of each agar plates was incubated aerobically or anaerobically and micro-aerophilically. After 7d incubation at 26°C the most dilute plate with distinct colonies was selected and the colonies

Fig. 11.3

Screening Method to Detect Bacteria Antagonistic to Salmonella typhimurium



wiped off the surfaces with a sterile cotton wool tipped swab. The bacteria were re-suspended in 50ml of N broth and screened as detailed in Fig. 11.3.

Thirty discrete colonies were also picked from each set of agar plates and inoculated into N broth and examined in the same manner.

The solution (I_3) which had proved inhibitory in Experiment 11.1 was also screened by this method.

Results

Six of the 45 solutions examined proved to be capable of inhibiting salmonellas viz:-

1. Aerobic and micro-aerophilic plate of litter L 44
2. Anaerobic plates oflitter L 46
3. Anaerobic plate of litter L 53
4. Anaerobic plates of litter L 54
5. Micro-aerophylic plates of solution (I_3) from Experiment 11.1.

No solutions derived from discrete colonies isolated from original agar plates proved to be inhibitory by this screening process.

(b) The Detection of Strains of Bacteria able to Inhibit Salmonellas by the Agar Disc Method (Experiment 11.9)

The bacteria from the solutions which had proved to be inhibitory to salmonellas in Experiment 11.8 were isolated and these cultures screened for their ability to inhibit salmonellas.

Experimental Details

One loopful of each culture broth was streaked on the surface of a previously dried plate of N agar, these plates were incubated at 26°C for 5d. Representative colonies were picked into N broth and these solutions incubated at 26°C for 5d after which 2ml of each solution was removed to a separate cell of a 25 compartment plate. A disc seeded with Salmonella typhimurium was placed in each compartment. After incubation at 26°C for 2d the discs were examined and the results recorded as described in the Materials and Methods section of this Chapter.

Results and Discussion

Fourteen of the 120 cultures examined were found to be inhibitory to salmonellas by this technique. The cultures were derived as follows:

Litter 46 - 4 cultures

Litter 53 - 5 cultures

Litter 54 - 2 cultures

Broth I_3 - 3 cultures

The small proportion of the original strains of bacteria examined suggest that very few bacteria from litter are able to inhibit salmonellas by direct antagonism even under laboratory conditions.

The screening procedure used up to this juncture may not have revealed mixtures of bacteria which are able to act in unison to inhibit salmonellas therefore such activity was examined in the next experiment.

(c) The Antagonism of Salmonella by Simple Mixtures of Bacteria Species found to be Non-inhibitory in "Pure" Cultures (Experiment 11.10)

One hundred and five of the strains of bacteria screened for inhibitory activity were proved negative. As already discussed it is possible that a mixture of strains which are non-inhibitory when grown would prove to be inhibitory. The number of combinations of mixtures which could be derived from 105 cultures is very large therefore only two-way re-combinations of cultures from each of the original plates will be considered.

Experimental Details

Each pure culture which had been proved non-inhibitory in Experiment 11.9 was inoculated separately into 5ml of N broth and these solutions incubated at 26°C for 3d. Using a grid layout the "pure" cultures from each original plate were re-combined in pairs by adding one drop of each culture to a fresh solution of N broth. After 5d incubation at 26°C, 2ml of each solution was removed and placed in a cell of a 25 compartment plate and one agar disc seeded with S. typhimurium added and incubated at 37°C for 24h. The disc was removed and the degree of growth scored.

The final pH value of the solution was determined and the level of ammonia assayed using Nessler's reagent.

Results and Discussion

When cultured in pairs many of the mixtures of these cultures were inhibitory to salmonellas either completely preventing growth

Table 11.4

Antagonism of Simple Mixtures of Bacterial Species to S. typhimurium as detected by Agar Disc Techniques

Culture identification					
b	I*** (8.6)				
c	I* (8.6)	PI* (8.6)			
d	PI* (8.6)	PI* (8.5)	PI* (8.4)		
e	I* (8.6)	PI* (8.3)	I* (8.4)	PI* (8.5)	
f	PI* (8.6)	I* (8.5)	I* (8.6)	I* (8.4)	PI* (8.4)
	a	b	c	d	e

Culture Identification

(pH value shown in parenthesis, Nessler Reagent * code 1

** code 2)

[See Chapter 2 D 2 (i)]

others were bacteriostatic. The results from one group of mixtures are presented in Table 11.4 and the complete results are presented in Table A 11 (ii) in Appendix 2.

In most cases there was also rise in alkalinity often linked with the presence of ammonia so these mixtures did not warrant further investigation.

(d) The Purification of Strains of Bacteria Anatagonistic to Salmonella typhimurium (Experiment 11.11)

The cultures used in the previous experiments (Experiments 11.9 and 11.10) were not correctly cloned bacterial strains therefore it was necessary to purify each culture before undertaking a more detailed investigation of each strain.

Experimental Details

Each culture of bacteria was purified by streaking one loopful of a broth culture on a previously dried plate of N agar and incubating at 26°C. The cultures were examined daily and when growth was considered complete three discrete colonies of each type of colony present was picked and streaked separately onto a fresh plate of N agar. This was repeated until each culture was assessed as pure on three successive transfers.

One colony from each plate was inoculated into a solution of N broth which was incubated for 5d at 26°C when 2ml of the solution was transferred to a cell of a 25 compartment dish and an agar disc seeded with S. typhimurium added. After incubation at 26°C for 24h the disc was removed and scored for the degree of growth, as already

described.

Results and Discussion

The "pure" cultures as originally isolated did in some cases prove to be mixed cultures, in particular some cultures were contaminated with a spore-forming bacteria which tended to spread over the surface of the plates and make the process of purification laborious. Not all the purified strains proved to be inhibitory therefore at the end of this process of selection 29 cultures remained which had been confirmed as antagonistic to bacteria.

(e) The Characteristics of the Pure Culture known to antagonise Salmonella typhimurium (Experiment 11.12)

This project did not aim to classify the species of bacteria indigenous to poultry litter but to investigate the inhibitory effect of these bacteria on salmonellas within the litter. However, at this juncture it was considered worthwhile to carry out a preliminary classification of the cultures involved in this phenomena.

Experimental Details

All cultures were examined for morphology by Gram reaction catalase test, motility, biochemical test by API system, oxidation-fermentation of glucose, aerobic decomposition of uric acid, liquification of gelatine, hydrolysis of hippurate, growth under anaerobic condtions and the appearance of the colony on N agar. Those colonies resembling coryneform bacteria were further subjected to tests for diastase production, haemolysis of horse blood, production of acid

from glucose in agar, and reaction in litmus milk.

Results and Discussion

The detailed results are presented in Table A 11 (ii) in Appendix 2.

By morphology 16 isolates could be identified as Coryneform bacteria, 10 as cocci and 1 spore forming bacteria. No Gram negative bacteria were identified.

The API method employed for the majority of the biochemical test was not designed for the use with strains such as those examined in this investigation, therefore it is not possible to be sufficiently confident in these results to subject them to detailed taxonomical arguements. Therefore the strains will be referred to as Coryneform 1-17, Coccus 1-10 and Bacillus sp. throughout this account. However from the available data, but taking into account the limitations of the accuracy of the tests, it is interesting to speculate the possible taxonomic position of the strains of the bacteria under consideration.

Comparison of the patterns of susceptibility of all the isolates to antibiotics shows that each culture isolated is a separate strain (Table A11 (iii) in Appendix 2).

Coryneform bacteria 1-16

The classification of the coryneform bacteria is uncertain (Goodfellow et al., 1981; Minnikin, 1981) however it is generally accepted that the genus Corynebacterium should be restricted to plant and animal pathogens (Bousfield, 1972; Jones, 1975) the non-pathogenic

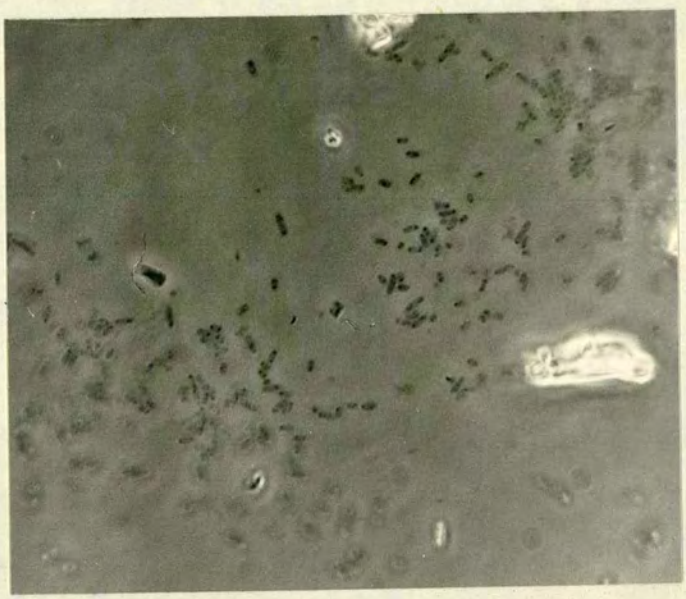
Photograph 11.5

Coryneform bacteria at various stages of morphological change.

(a) 6h

(b) 24h

(d) 7d



bacteria with coryneform morphology being included in the genus Arthobacter. In particular the numerical taxonomical studies of Jones (1975) strongly support this suggestion. The genus Brevibacterium is also of doubtful validity as the named species attributed to this group did not form distinct groups in the phenons of Bousfield (1972) and are listed as species incertae sedis in the latest edition of Bergey's Manual (Buchan & Gibbons 1974) although Jones (1975) considered that species Brevibacterium lineus, B. ammoniogenes and B. staticonis should be retained.

In this investigation the coryneform bacteria can be divided into those which are able to metabolise glucose aerobically and anaerobically namely strains 1, 3, 4, 10, 12, 14, 15 and 17, the remainder of which are relatively inactive.

The metabolically active strains may be classified as Arthobacters when the description in the 8th Edition of Bergey's Manual is consulted. However only Coryneform 1 may be identified to the species level resembling Arthobacter citreus. The remainder all of which do not liquify gelatine may be further divided by the ability to decompose urea, strains 10, 14, 15 and 15, being positive and the remainder negative. The latter group in many respects conforms to the species Arthobacter terregens, it not being possible to assign a species name to the remainder.

The inactive group generally conform to the characteristics designated to this genus by Jones (1975) and are similar to the isolates from poultry litter by Schefferle (1965). A comparison of the isolates from this study and those of Schefferle (1965) show

a great deal of variation only strains 3, 10 and 11 having similar metabolic profiles, conforming to Group C of her classification. Unfortunately the most recent examination of the cultures of Schefferle (1965) by Rogosa & Keddie (1976) has not been published in detail although they state that their results do support the original designation of her isolates.

Coccus 1-11

The classification of Gram positive cocci is equally uncertain. Coccus 2 and 9 which ferment glucose anaerobically may be considered in the genus Staphylococci. If these two strains are classified further according to the scheme of Baird-Parker (1963) Coccus 2 resembles Staphylococcus saprophyticus and Coccus 9 could be considered in Group IV of this classification although it does not conform completely to the biochemical profile of the group in that it does not utilise glycerol. The remaining strains in this group also by morphology appear to be Micrococci and not Aerococcus sp. According to the classification of Baird-Parker (1963) strains Coccus 5 and 8 conform to Group 5 while Coccus 1, 3, 4, 6, 7 and 10 fall into Group 7 although these isolates divert slightly from the profiles for these groups.

Spore forming Bacteria

The single strain of spore forming bacteria would appear to be Bacillus polymyxa when classified according to Gibson & Gordon (1974)

in the 8th edition of Bergy's Manual.(Buchan & Gibbons, 1974)

(f) The Establishment of Inhibitory Activity within Inoculated Solutions (Experiment 11.13)

The time which elapsed before inhibition could be detected by the agar disc assay was measured in the next experiment in order to establish the incubation time required in future experiments and to gain some information on the nature of the inhibitory agents.

Experimental Details

Sterile 10ml quantities of N broth were inoculated with each strain of bacteria and incubated at 26°C for 6h. After thorough mixing each solution was divided into 5, 2ml quantities in separate compartments of a 25 compartment dish and incubated at 26°C. One disc of Water agar seeded with Salmonella typhimurium were added at 0h and at 24h intervals for 5d. After exposure in the test solution for 24h, each disc was removed and the degree of growth coded as before.

Results

No inhibition was observed in the disc placed in the test solutions at 0h and 24h but after 48h all solutions inhibited the growth salmonellas in the discs.

These results show that the inhibition is probably due to the build-up of by products of metabolism which accumulate in the solution. Therefore all inoculated solutions were allowed to incubate for at least 2d before examination in the following experiments.

(g) The Ability of Pure Cultures to Inhibit Salmonella typhimurium in Four Non-selective Media (Experiment 11 .14)

The metabolism of bacteria and the resultant by products vary greatly depending on the growth media used. In this investigation to this point all inhibitory action has been detected in N broth which may not be the most favourable medium for the production of inhibitory agents. The following trial will investigate the presence of inhibitory agents when the selected strains are grown in four different growth media.

All solutions rendering a negative result were discarded without further investigation or confirmation and these observations must be considered as only preliminary in nature.

Experimental Details

Each strain of bacteria was inoculated into 100ml quantities of N broth, RCM broth, trypticase soya broth and BP water which was incubated at 26°C for 21d. Two ml quantities were transferred to a cell of a 25 compartment plate and one disc of Water agar seeded with Salmonella typhimurium added to each aliquot and the test solutions incubated for a further 24h at 37°C. The agar disc was removed to be assessed for growth as already described.

The solutions which allowed salmonellas to grow were discarded. The remaining solution which inhibited or partially inhibited salmonellas were centrifuged at 15,000 rpm for 60 mins to remove the bacteria. The supernatant transferred to two sterile vials. These broth supernatants will be referred to as the "supernatant" in the

following account.

The activity of the supernatants were characterised as follows:-

1) The activity against S. typhimurium using the seeded agar disc, untreated and after heat treatment at 121°C for 15 min.

2) pH value.

3) Presence of ammonia by titration of "supernatant" with pH level of over pH 8 (Chapter 2 D2b).

4) Antibiotic activity (Chapter 2 F).

5) Growth of S. typhimurium in "supernatant". 0.1ml of 10^{-2} dilution of an overnight culture of S. typhimurium was added to 5ml of each "supernatant" in a sterile test tube.

During incubation at 37°C the tubes were examined daily for visible appearance of growth. One loopful of each solution showing growth were streaked onto a dried plate of BG agar and these plates incubated at 24h at 37°C. The plates were then examined for the presence of colonies characteristic of salmonellas which were confirmed by the method described in Chapter 2 C.

Those solutions in which salmonellas did not grow were titrated to determine the concentration at which salmonellas could grow. This was achieved by diluting a fresh portion of the "supernatant" by factors of 1:1, 1:2, 1:4 and 1:16 with appropriate solutions and these diluted solutions challenged as before.

Table 11.5

The Procedure adapted for Treating Paper Chromatograms

Supernatant	Assay	Solvent	Detection*	Appearance
<pH 6	Lactic Acid	Ethanol (80): concentrated Ammonia (5): Distilled water (15)	Bromophenol blue reagent 1	yellow spots on a blue background
<pH 6	VFA's	Ethanol (85): concentrated Distilled water (10)	Bromophenol blue reagent 2	blue spots on a yellow background
>pH 7	phospho-lipids	Ethanol (80): Distilled water (10)	Exposed over Iodine solution	brown spots on white background
>ph 7	phospho-lipids	Chloroform (160): ethanol (40): Distilled water (5)	Exposed over Iodine solution	brown spots on white background

*Ingredients detialed in Appendix 1

6. Growth on Agar incorporating "Supernatant"

0.5g of Oxoid Agar-Agar No. 3 (L13) was added to 2.5-3ml of each "supernatant" in a ½oz. Bijou bottle. The mixture heated at 121°c for 15min and after cooling poured into 4cm dish. After setting and drying at 37°C for 30min the plates were surface inoculated with a loopful of an overnight culture of S. typhimurium in N broth. These plates were incubated at 37°C for 3d. The colonies growing on the surface were confirmed as salmonellas by slide agglutination test.

7. Identification of Compounds by Paper Chromatography

the supernatants were examined for the presence of a limited number of possible inhibitory compounds using the methods of Block et al. (1958) as detailed in the first section of this chapter using the reagents shown in Table 11.5.

Results and Discussion

The solutions showed varied degrees of inhibition as can be seen in Table A 11 (iv) in Appendix 2 no media being superior in their ability to enhance the inhibitory activity. After centrifugation the "supernatants" were showing the same activity as the complete solutions and therefore the original activity is present in the liquid phase and not the direct action of the bacteria in the solutions or on E_H effects. The inhibitory factor was heat stable in 83% of "supernatants" although there was a little loss of activity in 15% of "supernatants".

The inhibitory activity of the supernatants was not of an anti-

Table 11.6

The "Supernatants" inhibiting Salmonella typhimurium when incorporated in Agar

Culture	Media	Original pH	Agar Disc Assay
Coryneform 2	BPW	7.4	I
3	RCM broth	5.0	PI
4	RCM broth	5.9	PI
6	Tryptone soya broth	7.2	I
12	N Broth	7.5	PI

Table 11.7

Concentration of Ammonia in Alkaline Inhibitory Supernatants

Culture	Media	pH	Ammonia mg/ml
Coccus 4	N broth	8.0	0.35
Coryneform 9	N broth	8.1	0.30
Coryneform 6	N broth	7.6	0.38
Coccus 6	N broth	7.6	0.30
Control	N broth	7.2	0.16

biotic nature, no zone of inhibition being observed when impregnated filter paper discs were placed on seeded agar, and only 5 were inhibitory when incorporated in agar (Table 11.6).

It is noteworthy that in this situation Bacillus polymyxa which has been shown to produce an antibiotic active against salmonellas (Stansley et al., 1947) appears to be inhibiting these bacteria by another mechanism.

The inhibitory "supernatants" can be divided into three groups by their pH value i.e. those below pH 6, pH 6-7.5 and pH over 7.5. The supernatants in the latter group were titrated with sulphuric acid to determine the level of ammonia present. Table 11.7 shows that in these supernatants inhibition is due to the alkaline conditions and so were not further investigated.

The pH value of the supernatant resulting from the growth of each culture were compared, it was seen (Table 11.8) that growth of five cultures in the media chosen resulted in both neutral or acidic superantants.

When the ability of the "superantant" to support the growth of salmonellas was tested inhibition was only detected in eight "supernatants" (Table 11.9) but as can be seen the effect was diluted out by factors as low as 1 or 2 fold dilutions.

The investigation of a sample of the supernatant by paper chromtaography showed that the supernatants of acidic pH contained either VFA's or lactic acid (Photograph 11.3) while Coryneform bacteria 1, 4 and 8 and Coccus 2 contained substances of the phospholipid group.

Table 11.8

Comparison of Inhibitory Effect of Some Cultures in Different Media

Culture	Media with Inhibition Effect at	
	Neutral pH	Acid pH
Coryneform 1	Nutrient broth Tryptocase Soya broth	RCM broth
Coryneform 9	Nutrient broth BP water	RCM broth Tryptocase soya broth
Bacillus sp.	Tryptocase Soya broth BP water	RCM broth
Coccus 12	N Broth BP water	RCM broth

Table 11.9

The Inhibition of S. typhimurium by "supernatant" of Inhibiting broths as determined by titration

Designation of Culture	Media	Titre
Coryneform 1	RCM broth	1:1
Coryneform 9	Tryptocone soya broth	1:1
Bacillus sp.	RCM broth	1:1
Coccus 10	BP water	1:1
Coccus 12	RCM broth	1:1
Coryneform 3	RCM broth	1:2
Coryneform 4	RCM broth	1:2
Coryneform 9	RCM broth	1:2

These results show that the inhibitory effect of salmonellas by the products of bacteria derived from poultry litter is probably the result of a large number of mechanisms and would warrant a separate project. The extent to which the activity in laboratory broth is representative of that in the litter is always problematic. However as the inhibitory nature is present in the aqueous phase it is possible that such activity could take place in the litter. Throughout this investigation there has been evidence of microbial action interfering with the results the experiment involving poultry litter, the results of this experiment confirm this hypothesis. On the other hand if the criterion that the bacteria which inhibit salmonellas in litter are susceptible to streptomycin sulphate (Experiment 11.4) is used the only strains of bacteria investigated in this experiment which are possibly involved in inhibition in litter are Coryneform 16, Coccus 3 and Streptococcus faecium.

D General Discussion

It was unfortunate that it was not possible to set up a mixture of bacterial strains inhibitory to salmonellas in a chemostat but this failure indicated that these bacteria were anaerobes or facultative anaerobes. The establishment of such solutions in static solutions incubated in flasks suggest that the bacteria responsible were facultative anaerobes. Study of these static cultures initially indicated that the inhibitory activity was solely due to the production of ammonia which resulted in an alkaline solution. However statistical analysis of Experiment 11.3, showed that in a static

Photograph 11.3

Chromatogram developed to show the presence of volatile fatty acids.

culture derived from a 6w old litter it appeared that an additional mode of inhibition was being exerted. A further experiment (Experiment 11.4) of streptomycin sulphate, oxytetracycline and tetracycline showed that the bacteria responsible for the inhibitory action were susceptible to oxytetracycline and furazolidone but resistant to streptomycin sulphate, the reverse to the effect found *in vivo* with chicks treated to the "Nurmi concept". Beck & Chang (1980) showed that furazolidone is able to kill uric acid decomposing bacteria therefore it is possible that the inclusion of this antibiotic would destroy those bacteria responsible for the production of ammonia and so negate the inhibitory effect.

At this juncture it appeared that although bacteria were responsible for the inhibition of salmonella by the indirect method of degradation of nitrogenous components to ammonia which produced an alkaline environment which was hostile it was possible that other modes of inhibition were possible. But the further experiments confirmed the initial hypothesis of ammonia production being inhibitory mechanism and conventional plating techniques failed to detect bacteria able to act by any other inhibitory method. When the newly published agar disc method of MacFarlane & Makrides (1982) was used it was possible to determine bacteria able to antagonise salmonellas. The use of this technique showed that there appear to be a number of modes of inhibition. However it would appear that no bacteriocins are involved as no "antibiotic-like" activity was detected. It is interesting that Coryneforms 1, 4 and 9, Coccus 6 and *Bacillus sp.* were able to inhibit salmonella at

alkaline pH values in one broth and an acid pH in another medium as shown in Table 11.11. Theinhibiting effect of the acidic broths is probably due to the production of VFA's or lactic acid in the broth. Although this mode of inhibition could exert some effect in an immature litter it is not likely to be effective in the alkaline environment of a mature litter.

It is incertain which, if any, of these mechanisms of inhibition are active in litter. The numbers of strains involved would appear to be small and if the resistance of the bacteria to streptomycin sulphate is taken as a criterion of a strain involved in the inhibition in the litter (Experiment 11.4) only two of the cultures (Coryneform 2 and Coccus 10) under investigation could be considered as possibly relevant in litters; the accumulation of water soluble by-products of metabolism were possibly responsible. In laboratory media it is possible for the same strain to produce a variety of metabolic by-products depending on the formation of the basal medium. Five strains examined here have illustrated this phenomenon (Table 11.8). The inhibitory effect of the acidic broths was due to the production of VFA's or lactic acid and so are only of interest in relation to laboratory media and probably are not effective in the context of mature poultry litter.

The exact manner of the action of the agar disc method has not been published and as the technique has only recently been described there has been no scientific discussion on the method. In this investigation it would appear that the inhibitory substances diffuse into and accumulate within the disc and then prevent or disrupt the

growth of the cells of S. typhimurium. When the discs were assessed visually it was obvious that the partial inhibiton of the colonies was due to a number of factors, in some discs the colony formation was irregular, in some the colonies were small, while in other discs only some cells had developed forming disorganised colonies. This is possibly due to difference in the rate of diffusion or variations in susceptibility of individual cells of the test cultures of salmonellas. As synchronsied cultures were not used for this inhibitory test it is possible that variability within the stage of development of the cells in the test inoculum could account for some of this variability. When the discs were transferred to N broth some cells of S. typhimurium grew showing that the inhibitory substance diffused out of the disc, as at no time was there a lack of nutrients in the system. The demonstration of the effect both in broths with actively metabolising cells and the corresponding sterile "supernatant" indicates that the effect was not the result of a reduction in the oxygen tension in the broth.

The agar disc method relies on the diffusion of the inhibitory factor in a simple aqueous system and so it is probable that such modes of inhibition could exist in poultry litter. These results show that a number of modes of inhibition of salmonellas do exist within poultry litter and that this aspect should be further investigated.

SUMMARY

1. The bacteria responsible for the inhibition of salmonellas in poultry litter were facultative anaerobes.

2. These bacteria act either indirectly by degrading the nitrogenous compound in the litter to ammonia so producing a high level of alkalinity or directly by antagonistic activity.

3. In the litter there are probably very few bacteria able to antagonise salmonellas.

4. In the laboratory the mode of inhibition of salmonellas by bacteria isolated from litter was dependent on the culture medium.

5. The mechanism of inhibition of salmonellas by bacteria in litter is not similar to that of the "Nurmi concept" in chickens.

6. The group of bacteria responsible were Coryneform bacteria and Gram positive cocci the exact taxonomical position of the strains being uncertain.

CHAPTER XII

GENERAL DISCUSSION

GENERAL DISCUSSION

A Choice of Strain of Salmonella typimurium

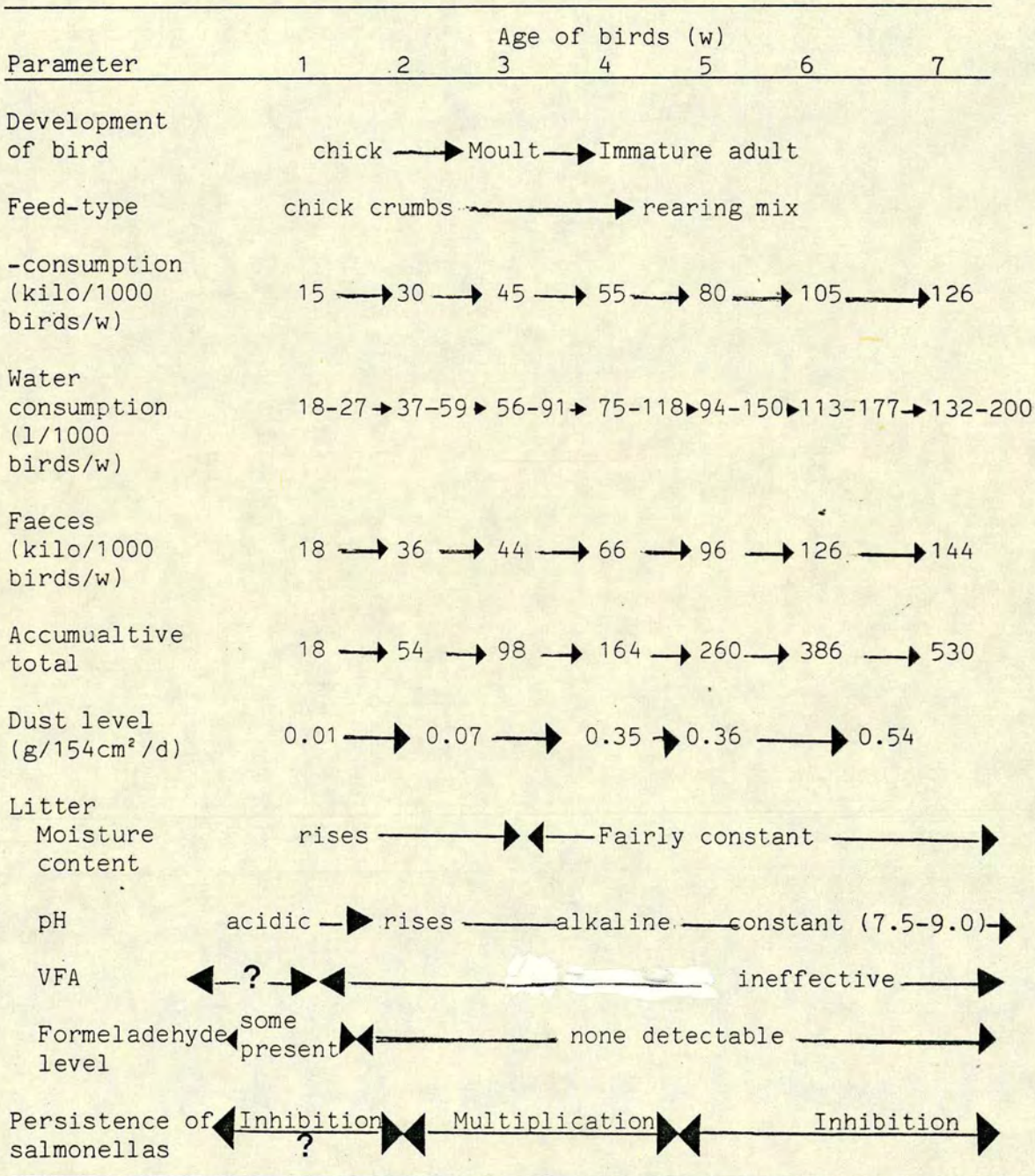
At the initial planning stage of this project it was decided to use a "wild" type strain of S. tyhphimurium rather than a multiple-resistant mutant although this involved devising a method of enumerating salmonellas in litter. The diversity of reactions encountered has justified the original decision for if these had been identified with a mutant strain it would have been necessary to confirm each effect with a "wild" strain. There still remains the possibility that the chosen strain of S. typhimurium could be atypical in some manner either when isolated or after cultivation in laboratory media. However such limitations are almost unavoidable in this type of investigation but circumstantial evidence indicates that no such change had taken place during the duration.

Although the decision not to use a mutant strain and to enumerate rather than detect the presence and absence of these bacteria has involved a great deal of work this has been justified as this method has allowed the persistence of salmonellas in litter to be studied in a much more detailed manner than would otherwise be possible.

B The Persistence of Salmonellas in Broiler Litter in Relation to the Age of the Flock

The presence of the birds on the litter appears to be critical in the formation of a mature litter as it is not possible to divorce the activity of the birds from the interaction of salmonellas with

Fig. 12.1 Time-flow diagram of some aspects of broiler birds over rearing period

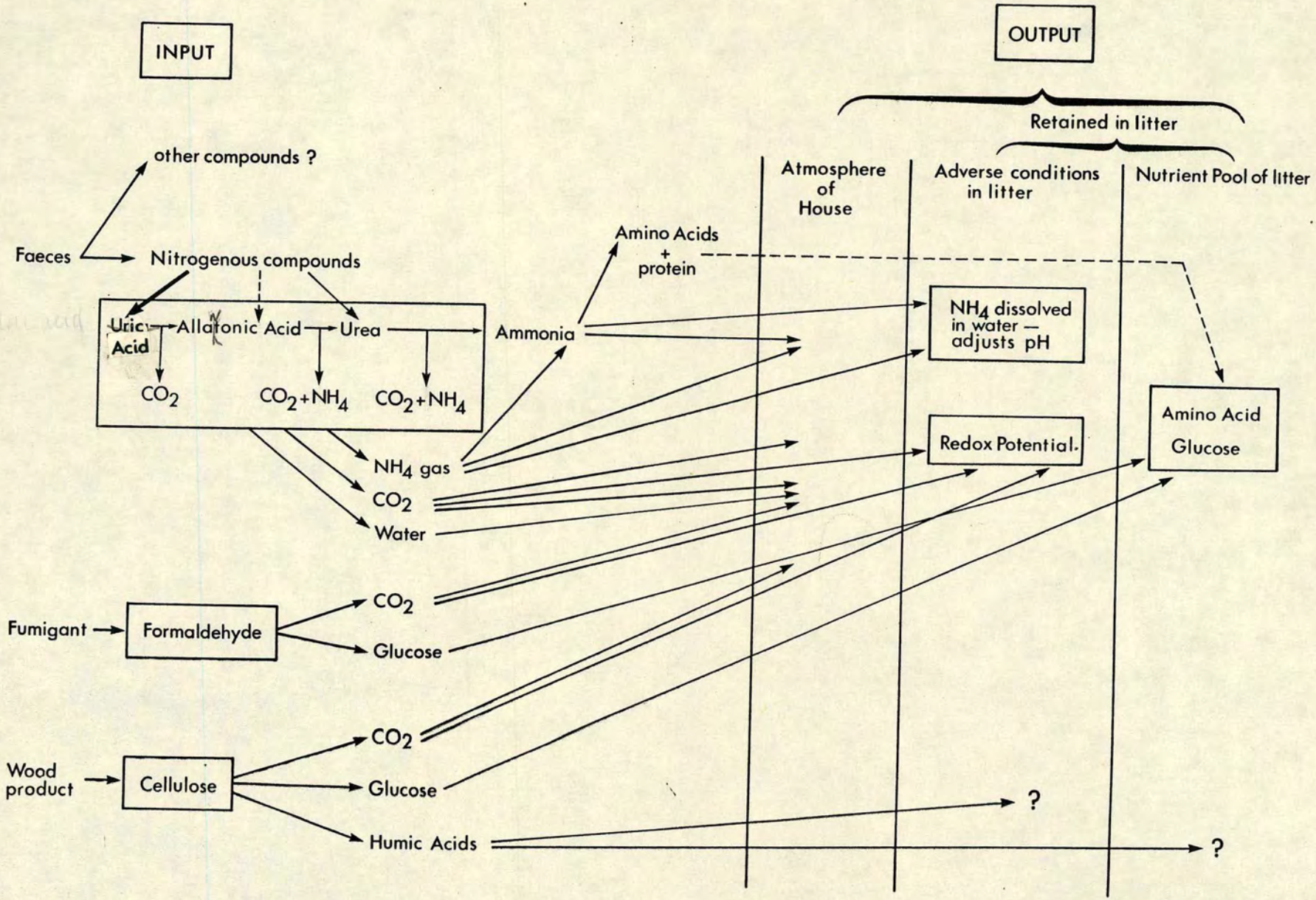


the litter material. Although attempts have been made to set up a "model" litter in the laboratory it was not possible to simulate the effect of the continuous presence of birds. While the loss of this type of experiment must be deplored it does underline that the dynamic situation of the broiler house is due to a large number of interacting factors.

The time-flow diagram of a broiler flock as seen in Fig. 12.1 shows that chicks when placed as "day-old" chicks weight about 1% of their final adult weight, with faeces containing few bacteria, feeding on chick crumbs which are rich in protein. Gradually the birds grow in size and shed increasingly large amounts of faeces which between 0-3w vary greatly with regard to the constituent species of bacteria, after 3w the adult gut flora has evolved and so the genera of bacteria shed in the faeces after this time are relatively constant (Barnes et al. 1972). In addition at 18-21d the birds moult and therefore the protein level will rise due to the incorporation of the feathers. Concurrently the feedstuff is changed to a rearing formulation with reduced protein content which in turn will lead to a change in the chemical constitutents of the faeces which are deposited in the litter.

The formation of a mature litter and the inter-relationships of conditions with an individual litter is complex and variable. In the absence of laboratory 'model' litter the interpretation of results must of necessity be based on circumstantial evidence together with the results of laboratory experiments in which no more than three parameters were manipulated in a single experiment.

FIG.12.2. FLOW-DIAGRAM OF MATERIALS WITHIN LITTER.



From the ecological standpoint a poultry litter can be described as an "open" system, that is a system into which material is constantly flowing and subsequently flows out in a modified form. From the literature a list of ingredients of the litters can be drawn up and the result of bacterial activity is anticipated it is possible to speculate a flow diagram in the litter (Fig. 12.2).

Initially formaldehyde in the litter prevents the growth of salmonellas (Chapters 7 and 8), the rate of dispersion varying greatly from house to house and so this cannot be relied upon to inhibit salmonellas (Chapter 8). Where the formaldehyde has disappeared from the litter the salmonellas deposited on the litter will multiply and so the litter can act as foci of contamination to the birds and may spread within the whole environment of the house. After 25-30d it would appear that the conditions in the litter stabilise and at about the same time the litter becomes potentially inhibitory.

The foregoing description has only described the evolution of the inhibitory effect in the litter but does not answer the question - why is mature poultry litter inhibitory to salmonellas? During the first phase as already stated inhibiton is due to the presence of formaldehyde in the litter, the level of inhibition being directly related to the amount of chemical in the litter. Although generally wood products per se do not contribute directly to this effect the variability between the species of woods to adsorb and later release formaldehyde does affect the length of time

that the mode of inhibition persists. Young litters are slightly acidic in nature and it is possible that VFA's could exert an inhibitory effect but the very small quantities present in the litter would make it unlikely that these chemicals are bacteriocides except in rare situations.

In a mature litter the mode of inhibition is of a more complex nature. The limited results from the litters collected at 72 poultry units (Chapter 7, Survey 1) it was considered that the moisture content, as determined by the conventional drying technique, was not related to the inhibition of salmonellas. In a later section (Chapter 8 C) it was established the critical factor was the water activity of the material as this gave a measure of the water available to the bacteria. However water activity and moisture content are not directly related so it is obvious that the moisture content and inhibitory activity are not necessarily correlated. An added complication is that water activity and persistence of salmonellas do not have a linear relationship. At high levels of water activity salmonellas multiply, at very low levels the cells survive, while at the mid-values these bacteria die out. There are no established scientific explanations for this phenomenon but it would appear that as the water activity falls from Aw = 0.6 to Aw = 0.4 the cells fail to survive. Further examination of the results revealed that even under controlled laboratory conditions the level of maximum inhibition varied and so while persistence may be related to water activity this is so difficult to quantify that it is not possible to anticipate the proportion of inhibition that is due to this

parameter however it would appear that the optimal water content for litter to exert the greatest inhibitory effect is 7.5-12.5%.

The pH level appears to be directly related to the inhibitory effect. It has been shown that after pH 8 the degree of inhibition gradually increased at the rate of 0.33 $\log_{10}$ per 0.1 units of pH but these bacteria were able to survive at pH 10 in a nutrient medium. The results of the survey confirmed that in poultry houses the litter gradually increases in alkalinity for the first 25-30d when the pH values remains constant at pH 7.8-9.0. In the litter it is the ammonia produced by the breakdown of uric acid and to a lesser extent urea which results in the increased alkalinity. In Chapter 8 section D it was established that it is the resultant pH level and not the presence of ammonia ions per se which resulted in this effect. Using identical methods it was possible to demonstrate that litter derived from mammalian faeces were the main nitrogenous excreta is urea behave in a identical manner (Chamore et al., 1981).

In the literature it is generally considered that the role of bacteria in the failure of salmonella to persist in mature litter is the indirect effect resulting from the rise in pH level due to the bacterial degradation of uric acid and urea. While this is undeniably the major contribution by bacteria to the inhibitory effect of litter this investigation has established that bacteria from litter can inhibit salmonellas by other by-products of their metabolism. It was not possible, in the time available, to establish the extent to which these compounds are present in the litter. Throughout this dissertation there have been inconsistencies in the results of

experiments where litters have been used as the basal medium which may be explained by assuming bacterial involvement. Direct antagonism between bacteria from the indigenous microflora and S. typhimurium could only be demonstrated by the agar disc method, as this appears to involve the concentration of the inhibitory factor in the agar disc. It appears that this factor is present in the aqueous phase so it is possible that this mode of inhibition could take place in poultry litter. It is noteworthy that the species of bacteria involved were all Gram positive bacteria viz. Coryneform bacteria, Bacillus sp., Streptococcus faecium, Staphyloccus spp. and Micrococci spp.

As the numbers of bacteria in the litter are very large it could be considered that the total load of microflora in itself could play a part in inhibiting salmonellas by direct competition for sites and nutrients. However it would appear that this is not so as the temperature of the litter and the reduced oxygen tension would appear to favour the growth of salmonellas. In addition comparison of litters with identical levels of total viable bacteria as determined by conventional methods show some to be inhibitory and some non-inhibitory.

Although not examined in the experimental work of this project the possibility of bacteriophage or protozoa contributing to the reduction of numbers of salmonellas should be considered. A preliminary experiment failed to isolate bacteriophage. However the inability to demonstrate these particles does not conclusively prove their absence as they may be difficult to demonstrate in some

materials. Prolonged exmaination of litter for bacteriophage was not considered warranted as the inhibitory effect of litter is not serotype specific as are the majority of salmonella bacteriophages. In addition, to be effective, the action of the bacteriophage should be by lysis. Similarly protozoa in the litter are likely to browse non-specifically on vegatative bacteria and as the numbers of salmonellas in the litter are likely to be small in comparison to other material effects this activity would be minimal.

While it is convenient to study parameters individually in the laboratory, this does not represent the situation in the litter where all the hostile parameters interact simultaneously. When discussing the inhibition of bacteria Leistner & Rodel (1981) suggest a "hurdle" theory in which they postulated that in order to survive bacteria must overcome a series of hurdles in their example, cooked meats they designate water activity, nitrate and brine levels as hurdles. They point out that in these studies they were unable to quantify the "heights" of the "hurdles" and so could not assess the relative importance of each parameter. In this investigation it was possible to apply this concept and to stepwise eliminate some of the parameters so that the magnitude of their effect can be calculated (Experiment 6.3). Hence it may be speculated that the 11% of the inhibition is due to water activity, a further 11% is removed by eliminating the solid material and 49% by negating the effect of the bacteria. However a further 28% of the inhibition remained unexplained.

The interaction of two or more parameters generally lead to the

total effect being the addition of the individual parameters except for temperature which did not affect the inhibitory effect. In litter no parameter acts in isolation and therefore the interaction may be more significant than shown by individual factors when trying to elucidate the total effect.

The use of a broth method to enumerate the salmonellas in the experiments of this investigation resulted in a final count which includes all viable cells. Similarly in the litter there will be salmonellas in a variety of metabolic states and some will be actively metabolising and multiplying, some while not physiologically damaged are not multiplying while others are physiologically damaged. The effect of the inhibition may be to initially damage the cells so that a futher adverse change in condition could lead to death. Similarly a change in condition which is favourable could lead to repair of the cells and so effect survival. The interaction of such parameters can be demonstrated with the results of the interaction of water activity and pH (Experiment 9.13) where the physiological state of the cells at each interaction was studied; while the levels of pH and water activity applied separately would not be expected to result in death of the cell, combining the parameters results in this effect.

In the context of the persistence of salmonellas in litter the concept of dormancy is important. There is new evidence that spore formers are not the only genera of bacteria to exist in a dormant state. In this investigation circumstantial

Table 12.1

Comparison of the Inhibition of salmonellas by the "Nurmi" Concept in the chick gut and in litter

Factor	"Nurmi" Concept	Litter
(a) Culture media	Not critical	Not critical
(b) Cultural atmosphere	Anaerobic	Aerobic or micro-aerophylic
(c) Faecal material	avian only	any species
(d) pH level	5.6-7.0	7.0-9.0
(e) Effect of anti-biotics		
- destroyed by	Furazolidone Oxytetracycline Streptomycin sulphate	Furazolidone Oxytetracycline -
Not destroyed by		Streptomycin sulphate

evidence would then tend to support this supposition. An example of this is the survival of salmonellas in a very dry situation it is difficult to explain the effect in terms of conventional theory that the maintenance of the population by the continuous multiplication of bactiera. It may be speculated that as the water activity is decreased over the range of water activity of Aw = 1.0 to Aw = 0.45 the cells are increasingly dehydrating and fail to survive but below the Aw = 0.45 it would appear that a state of "dormancy" is initiated so enabling the cells to survive. When placed in a medium of physiological affluence as in the broth of this investigation, the cells are able to multiply. If this phenomenon does exist it could have important implications in the interpretation of the results of many trials and observations pertaining to the longevity of infectious diseases in farm situations. Hence this aspect of microbial physiology would warrant further investigation.

As already discussed (¶1. A. 1.(ii)) the "Nurmi" concept is an established inhibitory effect in poultry. This effect involves the establishment of gut microflora and as the faeces form the active constitutent of the litter it is worthwhile to contrast both situations. The summary of the factors involved shown in Table 12.1 show that no similarities exist between the two inhibitory reactions. Therefore it must be concluded that in the litter it is the results of natural selection of the litter microbes from the chick faeces which in turn leads to the establishment of a totally different mode of inhibition.

In conclusion it would appear that the conditions even within

apparently identical poultry houses vary so greatly that each litter is unique and consequently it may never be possible to postulate a mode of inhibition of salmonellas in litter which is applicable to a large proportion of litters. However the data presented has identified the pH level and to lesser extent moisture content as probably the two most important parameters. It has also been possible to demonstrate that the indigenous microflora can act to inhibit salmonellas by degrading nitrogenous compounds to ammonia so raisng the pH value, but it has also been shown that there is some direct antagonism which was not identified.

Using these findings it may be possible to suggest modification to management methods both during the rearing of the birds and later during disposal of the litter. In this way the incidence of sub-clinical salmonellosis in the flocks could be reduced also the potential contamination of the environment could be averted.

C Practical Applications

The practical applications of this investigation in the future could be:-

1. Modification of the management of the litter onto which young chicks are placed.
2. Interpretation of the results of litter samples taken to determine the disease status of the flock.
3. Deposition of litter from houses which have held infected birds in a manner which is not hazardous to the environment.

Chicks are very susceptible to salmonella infection during the first few days of life and once infected can carry the disease subclinically and ultimately result in a contaminated carcase. Infected chicks coming to a clean house will quickly lead to salmonellas becoming established in the litter and later it can result in the whole house becoming contaminated. Management of the litter in such a way that it is inhibitory from the start of the rearing period could considerably reduce this build-up of infection. This investigation has shown that high pH levels have an adverse effect on the survival of the salmonellas, it has also been shown that although ammonia is responsible for this effect in the litter, addition of sodium hydroxide would achieve the same result. It is possible that the addition of urea to the litter could aid in maintaining the alkalinity as the chemical reaction between urea and sodium hydroxide results in the production of ammonia. An alternative method would be to maintain a relatively high level of formaldehyde in the litter but in practice this treatment would be opposed on health grounds by the farm staff as fear has been expressed that this gas has a carcinogenic effect. A third possibility is that the wood shavings could be seeded with a suitable bacterial culture. In practical terms it is difficult to envisage this method as a useful method as the bacteria involved would need to be resistant to low levels of formaldehyde and survive in a material with a low level of nutrients. However the treatment of the wood products prior to placement of the chicks would involve no capital cost

and be a simple procedure in farms using the spot brooder system as only a small area of this littered area would need to be treated. In particular it may be worthwhile changing to this system first at the broiler-breeder farms as the health of the broiler flocks is dependent to a great extent on the disease-status of the breeding flocks.

The rapid die-out of salmonellas in mature litters may explain the discrepancies found at commercial laboratories when litters are examined for the presence of salmonellas to determine the disease status of the flock. However it has been shown that although the numbers are reduced a low number will persist and therefore today with the sensitive methods that have been devised for the detection of salmonellas so long as a sufficiently large quantity is tested if present these bacteria should be isolated. When interpretating these results it must be remembered that the numbers of salmonellas present in no way represent the number in the litter at the time of collection.

Fig. 1.4 shows that disposal of contaminated litter could lead indirectly to the spread of these bacteria from the poultry farm to the general environment. While many litters are free of salmonellas there is always the problem of the handling of litters from houses where there has been an outbreak of salmonellosis however few clinical cases have been seen.

In this context it would appear feasible to treat the litter with sodium hydroxide to a pH of pH 10.5. This could be undertaken quickly and cheaply in the house the litter being sprayed with

sodium hydroxide solution or rotated and then the procedure repeated. After storage in the stack the litter could be used on the land in the normal manner.

D Conclusion

This investigation has shown that the interaction of salmonellas and the environment of poultry is complex. These results have shown three phases of persistence of these bacteria during the maturation of the litter and some modes of inhibition both physicochemical and biological. It has not been possible to suggest a model of inhibition although some quantification of the various aspects has been proposed. It has been possible to suggest some alteration in farm management procedures both within the broiler house and when disposing of the litter which could reduce the level of the load of infection to the birds or the contamination of the environment. However the exact nature of the ecology of salmonellas in poultry litter remains an enigma which due to the great variability of poultry litter may never be completely determined.

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ADDENDUM

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Appendix I A

Formulation of Media prepared from Ingredients

Unless stated all dehydrated ingredients supplied by Oxoid Ltd.; chemicals by BDH Biochemicals Ltd. or Sigma Chemical Co., antibiotics as Appendix II.

ADAS - Microbiology Departments of Agricultural Development and Advisory Service.

Cellulytic Agar (file of University of Edinburgh Microbiology Department)

Cellulose powder	5g
Peptone	5g
Yeast extract	1g
KH_2PO_4	0.6g
K_2HPO_4	0.4g
Agar-agar	1.2g
Litter extract	250ml
Distilled water	750ml

Cellulytic Broth (modified from Mann, 1968)

Basal Media	Bacto casitone	2.5g
	Bacto yeast extract	0.6g
	Litter extract	100ml
	Mineral solution A	150ml
	Mineral solution B	150g
	Resazurin (0.1%)	1ml
	Cysteine HCl	0.5g
	Sodium bicarbonate	4g
	Distilled water	600ml

Mineral Solution A	KH_2PO_4	3g
	$(NH_4)_2SO_4$	6g
	NaCl	6g
	$MgSO_4$	0.6g
	$CaCl_2$	0.6g
Mineral Solution B	K_2HPO_4	3g

Cellibiose, cysteine, sodium bicarbonate were prepared as a concentrated solution and autocalved separately at 121°C for 15 mins.

The broth was dispensed in 10ml quantities in 1.7cm tubes. A strip of Whatman's No. 1 filter paper dimentions approximately 10cm by 1cm added to each and the tubes closed with a rubber bung. Aseptic precautions were taken during the assembly of the tubes.

Ethyl Violet Azide Agar (Barnes & Goldberg, 1962)

RC Medium	38g
Agar-agar	12g

Prior to use the following was added to the molten media

Sodium azide 1ml 5% solution)
) Sterilised by Seitz filtration
Ethyl Violet 1ml 5% solution)

EYGA agar (Cure & Keddie, 1973)

Mineral base E	1000ml
Vitamin B_{12}	2mg
Yeast extract	1mg
Agar (Oxoid No. 1)	12g
Glucose	1g

The yeast extract and agar was added to 1 l of Mineral base E and warmed until agar dissolved. Glucose and Vitamin B_{12} added and the pH adjusted to 6.8. Sterilise at 121°C for 20min.

Stock Solutions

(400ml quantities, glass distilled water)

I	K_2HPO_4	80g
II	KH_2PO_4	62.4g
III	$CaCl_2$	2g
IV	$MgSO_4.7H_2O$	8g)
V	NaCl	2g)
VI	$(NH_4)_2SO_4$	20g

EDTA/Trace elements solution

EDTA	5g
$ZnSO_4.7H_2O$	2.2g
$MnSO_4.4H_2O$	0.57g
$FeSO_4.7H_2O$	0.5g
$CoCl_2.6H_2O$	0.16g
$CuSO_4.5H_2O$	0.16g
$Na_2MoO_2.2H_2O$	0.15g

Adjust to pH 6.0 with 40% KOH.

Mineral base E

Solution I	5.5ml
Solution II	4.5ml
Solution III	5.0ml
Solution IV	10ml

Solution V	10ml
EDTA/Trace elements	3.0ml

pH 6.8

Glucose Agar (Shefferle, 1957; 1965)

Glucose	5g
Peptone	5g
Lab lemco	5g
NaCl	2.5g
Bromocresol purple (1.6% ethanol)	1ml
Agar	12g
Distilled water	1000ml

pH 7.0

LICNR Broth (Hobden et al., 1973)

L-lysine	10g
Tryptone	5g
Yeast extract	3g
Mannitol	5g
Glucose	1g
Salicin	1g
Ferric ammonium citrate (brown)	0.5g
Sodium thiosulphate	0.1g
L-cysteine	0.1g
Neutral red	0.025g
Distilled water	1000ml

pH 6.2

M$_9$ broth (Gomez et al., 1973)

Na_2HPO_4	7g
Glucose	2g
NH_4Cl	1g
KH_2PO_4	3g
NaCl	0.5g
$MgSO_4$	0.25g
Distilled water	1000ml

pH 6.8

Malt Extract Agar (modified) (ADAS unpubl data)

Malt Extract Agar (Oxoid No. 59)	50g
Distilled water	1000ml

Prior to use the following was added to the melted media

Streptomycin	(500µg/100ml) 5mg of 0.1%	) Sterilised by
Penicillin	(250mg/100ml) 2.5ml of 0.1%	) Seitz filtration

NMR Broth (Whittenbury et al., 1970)

$MgSO_4.7H_2O$	1g
KNO_3	1g
$CaCl_3$	0.2g
Sequestrene ion complex (Geigy Johnson of Hendon Ltd.)	0.004g
Distilled water	1000ml

OAES Agar (Kauffman et al., 1963)

Glucose	5g

Yeast extract	2g
$NaNO_3$	1g
KH_2PO_4	1g
Sodium tauracholate	1g
Sodium propionate	1g
$MgSO_4$	0.5g
Agar Agar	20g
Distilled water	1000ml

Prior to use the following was added to the molten media

Streptomycin	(mg/100ml)	5ml of 0.1% solution	STERILISED BY SEITZ FILTRATION
Chloramphenicol	(mg/100ml)	2ml of 0.25% solution	

Oxidation/Fermentation MEDIA (Hugh & Leifson, 1953)

NaCl	5g
Tryptone	1g
Yeast extract	1g
K_2HPO_4	0.3g
Bromothymol blue	0.03g
Distilled water	1000ml

Prior to use 0.5ml of 10% Seitz filtered solution of glucose was adedd to each 5ml quantity of melted medium. The tubes were mixed by rolling between the hand to reduce aeration.

Peptone-yeast-extract Agar (Schefferle, 1957)

Peptone	2.5g
Yeast extract	2.5g
Agar	12g
Distilled water	1000ml

pH 7.0

Tetrathionate Broth (Rolfe, 1946)

Solution A

Iodine	200g
Potassium iodide	250g
Distilled water	1000ml

Dissolve a little potassium iodide in distilled water. Add Iodine and allow to dissolve before adding the remaining potassium iodide.

Solution B

Sodium thiosulphate	250g
Distilled water	1300ml

Preparation of Basal Medium -

Nutrient Broth No. 2(Oxoid)	25g
Ringer's tablets	2
Distilled water	1000ml

After dissolving the above mix 22ml of Solution A and 44ml of Solution B in a separate vessel ensuring that he solution is first decolourised and add 1ml 1% (aqueous) solution of BDH Brilliant Green. Mix and add to basal medium. Add approximately 100g Calcium carbonate and mix well before dispensing.

Thallous Acetate Tetrazolium Agar (Barnes, 1956)

Peptone	10g
Lab lemco	8g
Glucose	10g
Tri-phenyl tetrazolium chloride	0.1g
Agar Agar	12g
Distilled water	1000ml

pH 6.0

Prior to use 5ml of a 10% Seitz filtered aqueous solution of Thallus acetate was added.

½ strength Tryptone Soya Agar (ADAS unpubl. data)

Tryptone Soyal Agar (Oxoid CM131)	20g
Distilled water	1000ml

Prior to use the following were added to the molten medium

Cyclohexanine (1% aqueous)	5ml	) Sterilised by
Casein hydrolysate (20% aqueous)	10ml	) Seitz filtration

Uric Acid Medium (Stapp, 1920)

Yeast extract	2.5g
$Na_2HPO_4.12H_2O$	6g
Uric Acid	1g
Agar Agar	12g
Mineral solution	100ml
Distilled water	900ml

pH 7.0

Mineral Solution

K_2HPO_4	1g
$CaCl_2$	0.1g
$MgSO_4.7H_2O$	0.3g
NaCl	0.1g
$FeCl_3.7H_2O$	0.01g
Distilled water	1000ml

Plates are poured in two layers:-

Basal layer - as above

Top layer - basal agar with additon of 10g uric acid/100ml

Urea Decomposing Medium (Schefferle, 1957)

Peptone	10g
Lab lemco	10g
Distilled water	1000g

pH 7.0

After sterilisation the following were added:-

Cresol red (2% aqueous)

Urea (40% aqueous)

VK Agar (Barnes et al., 1972)

VL Broth (see below)	1000ml
Agar Agar	12g

Prior to use the following were added to the molten medium.

Kanamycin (1% aqueous)	5ml	Sterilised by Seitz filtration
Vancomycin (1% aqueous)	5ml	

VL Broth (Barnes & Impey, 1971)

Tryptone	10g
Lab lemco	2.4g
NaCl	5g
Cysteine HCl	0.4g
Glucose	2.5g
Agar Agar	0.6g
Distilled water	1000ml

pH 7.2-7.4

VL Agar (Barnes & Impey, 1971)

VL Broth (as above)	1000ml
Agar Agar	12g

VLhfl Agar

VL Agar	1000ml

Prior to use the following were added to the molten medium

Liver extract	50ml
Chicken faeces extract	50ml
Haemin solution	100ml

Appendix I B

1. Bromophenol blue reagent (Block et al., 1958)

Bromophenol blue	0.05g
Citric acid	2 g
Distilled water	100ml

2. Chlorophenol red reagent (Block et al., 1958)

Chlorophenol red	0.05g
Ethanol	100ml

pH 7.0 (adjusted with NaoH)

3. Hantzsch Reagent (Nash, 1953)

Ammonium acetate	75 g
Glacial acetic acid	1.5ml
Acetyl acetone	1ml
Distilled water	500ml

Allow to stabilize for 24h before use and discard at 7d. Stored at 4°C.

4. Nessler Reagent (Cowan & Steel, 1974)

Dissolve 5g potassium iodide in 5ml freshly distilled water, add a cold saturated solution of mercuric chloride until a slight precipitate remains permanently after thorough shaking. Add 40ml)N NaoH, dilute to 100ml with distilled water. Allow to stand for 24h before use. Do not shake prior to use.

Appendix I β

Extracts

(a) Litter Extracts

A 10% w/v suspension of litter in a conicle flask was shaken for 1min, allowed to stand at room temperature for 30 min, reshaken and then filtered through a double layer of butter muslin. The filtrate was then passed through a Whatman No. 1 filter paper and then sterilized by autoclaving at 121°C for 15min.

(b) Faecal Extract

Equal quantities of faeces from laying hens and distilled water were autoclaved at 121°C for 30min. The extracts were stored overnight at 4°C during which the sludge settled. The supernatant was decanted, the pH adjusted to pH 7.0-7.2 and then sterilized at 121°C for 15min (Barnes & Impey, 1970).

(c) Liver Extract

Twenty-seven grams of dehydrated liver extract (Difco Co. Ltd.) were added to 200ml of distilled water and held at 50°C for 1h. Thsi mixture was boiled for 1min, cooled and allowed to stand overnight at 4°C to allow the sludge to settle thereafter treated as described for the faecal extract (Barnes & Impey, 1970).

(d) Haemin solution

Dissolve 10mg haemin in 1ml of INNaoH and make up to 250ml. Autoclave at 121°C for 15min (Barnes & Impey, 1970).

Appendix I C

1. Bromephenol blue reagent (Block et al., 1958)

Bromophenol blue	0.05g
Citric acid	2 g
Distilled water	100ml

2. Chlorophenol red reagent (Block et al., 1958)

Chlorophenol red	0.05g
Ethanol	100ml

pH 7.0 (adjusted with NaoH)

3. Hantzsch Reagent (Nash, 1953)

Ammonium acetate	75 g
Glacial acetic acid	1.5ml
Acetyl acetone	1ml
Distilled water	500ml

Allow to stabilize for 24h before use and discard at 7d. Stored at 4°C.

4. Nessler Reagent (Cowan & Steel, 1974)

Dissolve 5g potassium iodide in 5ml freshly distilled water, add a cold saturated solution of mercuric chloride until a slight precipitate remains permanently after thorough shaking. Add 40ml)N NaoH, dilute to 100ml with distilled water. Allow to stand for 24h before use. Do not shake prior to use.

APPENDIX 2. DATA

Table A 3(i)

Comparison of the Range of Enrichment Broths and Plating Media for the Isolation of Salmonellas

Enrichment Broth	Number of Salmonellae isolated on:-				
	BG Agar	DC Agar	SS Agar	BS Agar	XCD Agar
Tetrathionate Broth (Kauffman, 1930)	<1.0	<1.0	<1.0	<1.0	<1.0
	0.95	<1.0	<1.0	<1.0	<1.0
	>3.04	<1.0	<1.0	<1.0	<1.0
	NA	<1.0	<1.0	>3.04	3.04
	>3.04	<1.0	<1.0	<1.0	<1.0
	>3.04	<1.0	<1.0	>3.04	3.04
	>3.04	>3.04	0.84	>3.04	<1.0
	2.38	>3.04	<1.0	>3.04	1.38
Tetrathionate Broth (Hafman & Damon, 1956)	<1.0	<1.0	<1.0	<1.0	<1.0
	0.78	<1.0	0.6	<1.0	<1.0
	>3.04	>3.04	<1.0	<1.0	<1.0
	1.88	1.18	<1.0	<1.0	<1.0
	>3.04	<1.0	<1.0	<1.0	<1.0
	0.6	1.3	1.3	<1.0	<1.0
	<1.0	<1.0	<1.0	<1.0	<1.0
	<1.0	<1.0	<1.0	<1.0	<1.0
Tetrathionate Broth (Heard et al., 1969)	<1.0	<1.0	<1.0	<1.0	<1.0
	<1.0	<1.0	0.6	<1.0	<1.0
	>3.04	>3.04	<1.0	<1.0	<1.0
	1.88	1.18	<1.0	<1.0	<1.0
	3.04	<1.0	<1.0	<1.0	<1.0
	0.6	1.3	1.3	<1.0	<1.0
	<1.0	<1.0	<1.0	<1.0	<1.0
	<1.0	<1.0	<1.0	<1.0	<1.0
Selenite Broth (Oxoid)	<1.0	<1.0	<1.0	<1.0	<1.0
	<1.0	<1.0	<1.0	<1.0	<1.0
	1.72	<1.0	2.4	<1.0	<1.0
	<1.0	<1.0	<1.0	<1.0	<1.0
	>3.04	<1.0	<1.0	<1.0	<1.0
	>3.04	<1.0	<1.0	<1.0	<1.0
	1.40	<1.0	2.46	<1.0	<1.0
Selenite-Cysteine Broth (North & Bartram, 1975)	<1.0	<1.0	<1.0	<1.0	<1.0
	1.32	<1.0	0.6	<1.0	<1.0
	0.48	<1.0	2.4	<1.0	<1.0
	<1.0	<1.0	<1.0	<1.0	<1.0
	0.95	<1.0	<1.0	<1.0	<1.0
	<1.0	<1.0	<1.0	<1.0	<1.0
	<1.0	<1.0	<1.0	<1.0	<1.0
	<1.0	<1.0	<1.0	<1.0	<1.0
LINCR Broth (Hargrove et al., 1971)	>3.04	<1.0	0.6	<1.0	<1.0
	2.4	>3.04	<1.0	1.32	<1.0
	>3.04	>3.04	>3.04	<3.04	<1.0
	>1.0	<1.0	2.98	<1.0	<1.0
	<3.04	1.32	<1.0	2.18	1.43
	1.95	<1.0	0.78	0.6	<1.0
	<3.04	1.38	>3.08	<1.0	<1.0
	>1.0	1.88	<1.0	1.88	<1.0

Table A 3(ii)

The number of some Salmonella serotypes determined by the Plate Count on Nutrient Agar and MPN-10 method with LICNR Both containing Extracts of Poultry Litter(s)

Experiment No.	Serotype	Extract of Litter	Number of Salmonella isolated by: LICNR Broth	Plate Counts
1	S. agona	L12	8.46	8.39
	S. typhimurium	L12	8.60	8.39
	S. worthington	L12	8.46	7.82
2.	S. agona	L16	7.79	
	"	L17	7.52	6.59
	"	L18	7.60	
3.	S. typhimurium	L16	7.69	
	"	L17	7.90	7.39
	"	L18	7.06	
	S. worthington	L16	7.69	
		L17	7.79	6.48
		L18	7.69	

Table A 3(iii)

Comparison of number of Salmonella typhimurium in artificially inoculated poultry litter using a method incorporating a resuscitation phase and LICNR Broth

Litter Number	Inoculum	Presumptive count of salmonellas by LICNR Broth	Presumptive count of salmonellas by Resuscitation Method
L19	*S. typhimurium* (6.87/g)	7.26	6.98
L20		6.98	5.74
L21		6.54	6.54
L22		6.40	6.40
L23		6.95	6.74
L24	*S. worthington* (6.48/g)	6.48	6.74
L25		7.20	6.74
L26		6.91	6.43
L27		7.20	4.76
L28		7.20	4.74
Mean ± S.D.	6.65±0.21	6.91±0.32	6.18±0.82

Table A 3(iv)

The reliability of the colour change of LICNR Broth as indication of the presence of Salmonellas in solutions (360 tubes)

Sample Number	MPN salmonellas/ml by colour of LICNR Broth	MPN salmonellas/ml by plating on BG Agar	No. of false results* positive	No. of false results* negative
1	6.25	6.54	1	2
2	7.42	6.30	3	1
3	6.44	7.43	3	3
4	6.24	6.44	2	1
5	7.54	7.54	1	1
6	4.30	5.96	0	7
7	>7.26	>7.26	0	0
8	5.54	5.54	1	1
9	4.30	5.96	0	6
10	>7.26	>7.26	0	0
11	6.44	7.20	0	2
12	6.74	6.96	0	2
13	6.96	>7.26	0	2
14	6.35	6.74	0	1
15	6.34	6.54	0	1
16	6.34	6.40	0	0
17	6.96	6.96	0	0
All Samples			11	30
% False results			3	8.3

* Presence on BG Agar taken as positive isolation.

Table A 4(i)

Number of Salmonella typhimurium isolated from samples of varying weight inoculated with equivalent numbers of cells

Sample weight	sub sample No.	No. salmonellas isolated from litter:- L29	L30	L31	L32
25g	1	<1.00	3.36	7.36	8.18
	2	1.63	3.63	8.63	7.97
	3	4.36	3.63	9.18	7.63
	4	1.36	2.63	7.63	7.63
	5	>1.00	3.97	5.36	7.36
10g	1	<1.00	4.38	7.63	6.88
	2	0.95	4.63	>10.00	7.36
	3	0.69	3.38	9.32	8.18
	4	<1.00	3.97	7.30	7.63
	5	0.60	2.36	>10.00	8.18
1g	1	<1.00	2.60	>10.00	7.97
	2	1.36	2.36	7.36	7.97
	3	<1.00	4.63	NA	8.36
	4	<1.00	2.88	5.18	7.36
	5	0.60	3.18	>10.00	7.18

Table A 4(ii)

The variation of microbial and physical parameters litters at Sampling Stations at Three Houses

(a) Persistence of Salmonellas

House No.	Sampling Station	No. of salmonellae isolated in replicates:- 1	2	3	4	5
1	A	4.88	4.88	7.63	6.88	9.36
	B	4.63	5.38	>10.04	7.97	9.97
	C	3.95	>10.04	5.63	>10.04	6.36
	D	4.36	6.97	5.59	8.97	8.36
	E	6.36	3.88	6.88	>10.00	8.18
2	A	4.32	4.63	3.63	5.95	5.60
	B	4.36	3.36	1.36	1.36	1.36
	C	4.63	4.97	3.97	4.18	NA
	D	4.97	3.63	3.36	3.88	2.63
	E	0.60	1.36	0.95	2.63	1.36
3	A	1.36	<1.00	<1.00	<1.00	5.36
	B	1.36	1.98	2.36	1.85	2.18
	C	3.97	2.95	1.36	2.85	1.85
	D	3.36	1.36	3.36	3.60	1.88
	E	2.63	2.36	<1.00	3.36	NA

Table A 4(ii)

The variation of microbial and physical parameters litters at Sampling Stations at Three Houses

(b) Number of Aerobic Bacteria/g

House No.	Sampling Stations	No. of Aerobic bacteria isolated in replicates:- 1	2	3	4	5
1	A	7.20	7.59	6.00	7.54	9.44
	B	7.58	7.90	7.20	7.20	10.84
	C	9.61	8.05	9.69	10.04	10.78
	D	10.84	10.69	10.69	11.00	10.37
	E	10.24	10.64	10.41	10.95	11.00
2	A	10.52	10.48	10.95	10.22	10.12
	B	10.52	10.46	10.46	10.33	10.67
	C	10.07	10.60	10.59	10.49	10.41
	D	10.63	10.65	10.52	11.18	10.46
	E	10.58	10.56	10.55	10.56	10.62
3	A	10.95	10.30	10.54	10.88	10.36
	B	10.76	10.79	10.38	11.30	10.78
	C	10.48	10.69	10.37	10.36	10.15
	D	8.12	8.36	8.68	9.69	8.45
	E	8.02	8.62	8.07	8.33	9.70

Table A 4(ii)

The variation of microbial and physical parameters litters at Sampling Stations at Three Houses

(c) Number of Aerobic Bacteria/g

House No.	Sampling Station	No. of Aerobic bacterial isolated in replicates 1	2	3	4	5
1	A	5.27	6.44	6.30	6.90	6.83
	B	6.04	7.32	5.46	5.34	5.62
	C	9.38	7.80	7.84	9.59	10.17
	D	10.29	10.79	10.45	10.45	9.20
	E	9.78	9.97	9.93	10.78	10.95
3	A	9.69	8.61	8.76	8.51	8.46
	B	8.23	8.72	7.85	9.69	8.99
	C	8.32	7.72	8.15	7.85	8.64
	D	10.42	10.72	10.52	10.61	10.58
	E	10.91	10.78	10.92	10.99	10.90

Table A 4(ii)

The variation of microbial and physical parameters litters at Sampling Stations at Three Houses

(d) pH value of litter

House No.	Sampling Station	pH value of litter in replicates 1	2	3	4	5
1	A	5.10	4.90	5.20	4.80	4.90
	B	5.00	4.90	4.90	5.00	5.00
	C	6.10	5.90	6.10	6.00	6.10
	D	6.60	6.70	6.30	6.30	6.70
	E	7.60	7.30	7.00	7.60	7.20
2	A	7.70	8.20	8.20	7.85	7.95
	B	8.08	8.05	8.10	8.15	8.10
	C	7.50	7.85	7.55	7.60	7.65
	D	7.85	8.10	8.15	8.00	8.00
	E	8.10	8.20	8.00	8.05	8.20
3	A	7.36	7.60	7.25	7.35	7.10
	B	7.65	7.50	7.40	7.65	7.40
	C	7.65	7.60	7.60	7.65	7.30
	D	7.60	7.75	8.25	8.20	8.10
	E	7.90	7.95	7.95	7.45	7.40

Table A 4(ii)

The variation of microbial and physical parameters litters at Sampling Stations at Three Houses

(e) Moisture Content (%) of litter

House No.	Sampling Station	Moisture Content % in replicates 1	2	3	4	5
1	A	16.10	18.33	16.16	26.90	17.30
	B	21.50	21.21	17.50	20.00	19.81
	C	12.44	18.83	13.41	16.13	16.00
	D	25.80	22.07	17.74	NA	22.34
	E	25.85	33.70	29.76	23.23	31.40
2	A	27.41	23.39	24.41	20.00	24.28
	B	26.22	25.22	25.02	29.26	27.38
	C	23.48	22.24	24.97	22.85	22.74
	D	23.76	24.84	28.68	23.79	23.31
	E	25.55	28.86	24.40	23.83	13.95
3	A	28.17	29.65	30.98	26.69	33.51
	B	34.49	30.88	NA	22.68	24.98
	C	27.16	25.68	21.39	21.93	18.60
	D	20.03	30.50	31.66	34.48	34.50
	E	27.68	26.07	30.74	27.22	33.99

Table A 5(i)

The Chemical Analysis of Wood Shaving and Sawdust cited in the Literatures (%DM)

Parameter \ Author(s)	Labasky et al. (1977) a	Labasky et al. (1977) b	Choong (1967) a	Choong (1967) b	Collison (1976)	Carter (1979)
Crude Protein					22.5	
Nitrogen	0.2	0.1	3.7	3.4	26.6	
Crude Fat			2.8	2.7	1.6	
Ash	0.2	0.4	14.8	13.2	22.3	
Carbohydrate	67-71	67-71				
Acid detergent fibre			40.1	41.4		
Lignin	27-30	24-26				
Phosphate			1.8	1.9		
Calcium			2.2	1.7		
pH	5.1	5.3	6.8	7.0		
Moisture content					20.30	42.2-50.8
Wood species	softwood	hardwood	hardwood	Pine		

Table A 5(ii)

(a) Chemical Analysis of Poultry Faeces as cited in literature (mg/g)

Parameters \ Author	Hennig et al (1975)	Collison et al (1976)	Evans (1978)	Hamblin (1980)	Patkowska & Sholo (1980)	Poldori (1972)	Joyd & Misra (1971)	Shannon et al (1973)	McNabb et al (1975)
Organic matter									
Crude protein	33	345-404				196.60	335.6	5.1	54.9
True protein									
Ammonical nitrogen									
Non-protein nitrogen	12.1						47.85 of total		
N-free extract		231-				328.7	280.9		
Crude fat		5-22	15-21			41.4	40.1		
Ash		191-270	171-299	322-373		291.8	217.9	336	204
Water soluble carbohydrate								7.6	10.7
Crude fibre		89-114		99-182		141.5	125.5		
Detergent fibre			147-214						
Total phosphate			14-175	21-28	1.1	16.4	19.2	9.5	6.0
Calcium								9.5	

Table A 5(ii)

(b) Nitrogen Compounds in Poultry Faeces as cited in literature

Compound \ Author	Squance (1966)	Messer et al (1971)	Mengalli et al (1972)	Poldori (1972)	Bohme et al (1973)	Salter et al (1974)	McNabb et al (1975)	Okumura et al (1978)	Beck & Changi (1980)	Krogdahl & Dalsgaard (1981)
Total nitrogen	77.25			59.7						
Soluble nitrogen								4.8		
Soluble non protein N								21.8		
Uric nitrogen	51.03	5.0-17.0	10.5	22.8	1.8-40.9	196-215	55-72		99-156	not detected
Urea nitrogen	0.741	16.0								0.5-0.6
Ammonia	1.724	5.4-14.3		5.8-32.5		11-21				1.3-1.7
Creotinine nitrogen	2.60					2-11				
Unit	mg/g DM	mg/kg		%	% Total N	mg/gN	%	mg/d	mg/100g	% N

Table A 5(ii)

(c) Levels of Amino acids in Poultry Faeces as cited in literature

Author	Feldofer et al (1974)	Wilkie & van der Merwe (1974)	Bhargaya & O'Neil (1975)		Feldofer et al (1975)		McNaBB et al (1975)	Sibbald (1979)	Obumura et al (1978)	Webb (1979)	Kessler (1981)
			a	b	a	b					
Lysine	0.74	0.289	0.76	0.93	28.2	24.8	5.4	21.40	20.8	1.8	23.60
Proline							-	24.4	-		21.99
Alanidine	1.05	0.546	0.92	1.09			8.2		37.6		
Glycine	0.85	0.705	1.37	1.49	28.4	38.4	6.6		29.9	5.2	
Threonic		0.449	0.78	0.80			5.3	20.8	39.5	1.4	20.69
Aspartic acid	1.31	0.787	0.99	1.50	22.0	29.3	6.1	30.2	68.4	4.4	30.64
Valine	0.72	0.422	0.76	1.10	22.6	26.4	4.9	17.0	51.0	2.5	18.39
Alanine					21.9	24.3	-	17.0	-		16.15
Arginine		0.297	1.45	1.22			6.1	20.6	16.1	1.9	17.13
Glutomic Acid	2.19	1.078	4.60	2.45	14.2	18.7	16.4	48.4	201.2	6.4	49.00
Histidine		0.176					3.0	12.0	9.4	0.6	13.17
Isoleucine	0.55	0.364	0.64	0.59			3.8	13.0	59.4	1.4	13.18
Leucine	1.39	0.592	1.22	1.23	21.1	23.4	70	25.5	37.5	2.7	20.52
Methionine	0.38	0.111	0.47	0.62	15.8	18.9	1.2	5.0	11.8	0.5	5.51
Phenylalanine		0.357	0.72	0.80	17.1	16.7	3.9	12.2	17.6	1.3	11.37
Lecine		0.484	0.85	1.15	16.9	17.9	-	26.6	-	1.9	24.2
Tyrosine		0.129	0.73	0.58	21.2	20.9	3.4	15.0	21.4	0.6	13.02
Cystine			0.47	0.26			1.8		33.7	1.0	
Proline	0.87	0.631	2.26	1.25	19	24.1	-	24.4	64.8	1.4	21.99
Tryphosphin										1.9	
Treonin	0.8				25.3	33.4					
Hidrohsipolin	0.16				30.9	-	6.2		120.9		
Serin	0.83										
Units	g/day	g/100 DW	% DM		NOT STATED		DM%	mg/bird/ 24h	g/160g	g/16g N	mg/bird/ 24h

Table A 5(ii)

(d) Minerals isoalted from Poultry Faeces as cited in literature (mg/g)

Element \ Author(s)	Ivos et al (1966)	Polduri (1972)	El-Sakkon (1969)	Wilkie & van der Merwe (1974)	Smith (1974)	Evans (1978)	Hamblin (1980)	Patkowska-Sabola (1980)
As		0.94	11				0-4	
Ca		3.6		11.8		5.1-9.0	5.6-11.1	5-5.8
Cd							0.1-0.3	
Co		0.35	1.73					
Cu		3	98	0.048	7.3-8.3	21.32	47-94	1.4-2.5
Fe		2200	4510	2.87		0.5-1.01	0.1-0.4	10.8-11.2
Mg			0.44	1.13		0.53-0.55	0.4-1.03	1.1
Mn		230	225	0.88		0.22-0.23	0.2-0.45	7.5-10
Mo								1-1.5
Na		0.55	0.54			0.15-0.18	0.1-0.96	0.36-0.38
Pb							0.1-2.96	
Po	1.75	2.04	1.78	1.51		1.73-1.86	1.7-7.3	2-2.7
Se							0.001-0.01	
Si								
Zn			235	1.01		0.23-0.27	0.21-0.45	12.5-25

Table A 5(ii)

(e) Volatile Fatty Acids in Poultry Faeces as cited in literature

Acid \ Author(s)	Shrimpton (1963)	Rys & Koschoski (1974)	Watanabe (1975)
Acetic Acid	53		62.9
Propionic Acid	27		25.86
iso-butyric Acid	11		3.99
n-butyric Acid			10.48
iso-Valeric Acid	8		5.15
n-Valeric Acid			5.88
Methylanolic Acid		36	
pH	6.2-7.8		
Origin	Caecum		Gut contents
Unit	Molecular proportion in 20% solution	mg/chick/day	Total mg/g

Table A 5(ii)

(f) Chemical in Faeces originating from Medication as cited in literature (ppm unless stated)

Chemical \ Author	Caswell (1977)	Hamblin (1980)
Aflatoxin		0-25
Amprolicium	1.89	
Arsenic		
Chlortetracycline		0-0.04
Erythromycin		0-0.2
Neomycin		0-1.0
Oxytetracycline		0-0.16
Penicillin		0-0.04
Streptomycin		0-0.8
Sulfaqunoxidine	7.13	
Tetracycline		0-0.2
Zinc bacitracin		0.095 unit/g

Table A 5(iii)

The persistence of Salmonella typhimurium in hen faeces fed on 6 different diets

Cage No.	Number of Salmonellas persisting (g)	Feed No.	pH
1A	2	6	7.00
1B	1	4	7.00
2A	0	6	6.00
2B	0	4	6.50
3A	4	2	6.00
3B	4	2	6.00
4A	5	2	7.50
4B	1	2	8.00
5A	0	5	6.00
5B	3	6	8.00
6A	0	5	6.50
6B	2	6	NA
7A	0	3	6.00
7B	0	1	NA
8A	2	3	6.50
8B	0	1	NA
9A	0	1	6.00
9B	1	5	6.50
10A	0	1	6.00
10B	6	5	7.50
11A	6	4	6.80
11B	3	3	8.00
12A	6	4	7.20
12B	1	3	6.50
13A	3	5	7.00
13B	0	1	6.00
14A	3	5	8.00
14B	0	1	6.00
15A	0	2	6.50
15B	6	4	6.00
16A	6	2	7.00
16B	0	4	6.00
17A	0	3	6.50
17B	2	6	7.00
18A	0	3	6.50
18B	1	6	7.50
19A	2	4	6.00
19B	0	3	7.50
20A	0	4	NA
20B	0	3	6.50
21A	1	1	NA
21B	1	2	NA
22A	0	1	7.00
22B	0	2	6.00
23A	1	6	8.00
23B	0	5	NA
24A	6	6	NA
24B	0		NT
Feedstuffs 1	6		NT
2	6		NT
3	6		NT
4	6		NT
5	6		NT
6	6		NT

Table A 6(i)

The persistence of Salmonella typhimurium in poultry litter treated to demonstrate the relative importance of the various extracts

Extracts		No. of salmonellas isolated at		
Litter No.	Treatment	6h	24h	48h
L39	None	4.38	1.84	2.36
L40		3.38	1.60	1.60
L41		3.38	>1.00	>1.00
L42		3.59	2.30	>1.00
L43		3.60	2.97	0.85
L44		1.00	6.48	1.04
L45		6.36	1.48	1.36
L46		6.36	2.63	1.36
L47		6.36	2.97	1.36
L48		4.17	NA	NA
L49		3.60	NA	NA
L50		1.70	NA	NA
L39	Aqueous extract	5.08	3.66	1.30
L40		4.08	3.04	1.08
L41		3.08	3.04	2.34
L42		6.18	3.04	2.38
L43		5.96	4.38	3.38
L44		5.96	4.38	4.38
L45		6.18	3.38	1.48
L46		5.97	3.32	1.36
L47		5.97	2.30	1.59
L39	Seitz filtered	6.08	6.04	6.08
L40		NA	NA	NA
L41		4.0	8.04	6.08
L42	Filtered	6.36	4.38	1.38
L43	(Whatman No. 1)	6.36	4.38	1.04
L44		6.36	2.97	1.72
L45		6.36	2.97	1.63
L46		6.36	3.66	1.30
L47		6.36	3.66	2.04
L42	Seitz filtered	4.08	7.18	8.08
L43		7.08	7.36	9.08
L44		5.66	5.66	4.08
	Control 1 (L39-42)	6.38	9.66	10.38
	Control 2 (L43-44)	7.18	9.38	9.38
	Control 3 (L45-47)	7.17	9.36	9.18

Table A 7(i)

The persistence of Salmonella typhimurium in litter from commercial houses after inoculation at the laboratory

Enterprise	Age of Litter (d)	Number of Salmonellas persisting/g	M.C.(%)	pH
1	1	1.36	NA	5.80
	7	0.96	NA	5.90
	14	3.18	NA	7.05
	21	5.00	NA	8.20
	27	2.18	NA	8.40
	36	2.97	NA	8.50
	44	<1.00	NA	7.90
	53	1.36	NA	8.10
	56	0.60	NA	8.35
2	21	<1.00	27.3	7.95
	22	NA	36.76	7.75
	22	4.04	15.41	8.80
	39	6.38	34.47	7.95
	39	2.63	48.71	8.50
	38	3.60	25.51	8.50
	4	6.46	25.31	7.35
	6	6.63	NA	6.80
	7	6.95	25.83	7.00
	8	<1.00	22.34	6.25
	8	4.63	36.70	6.70
	11	4.97	15.52	NA
	11	5.66	NA	6.60
	12	5.66	20.60	8.45
	14-15	6.63	27.62	6.25
	15-18	6.63	23.20	7.90
	18	4.63	36.83	8.10
	19	4.63	24.08	8.65
	35	1.66	25.50	8.55
	34	1.48	22.10	8.55
	38	<1.00	27.55	8.55
	38	<1.00	26.17	8.45
	39-42	1.48	28.43	8.85
	42-45	<1.00	13.89	8.15
	45-46	1.48	NA	8.85
	48	2.36	44.98	8.80
	48	<1.00	22.59	8.30
	60	2.60	23.15	8.40
	55	3.38	10.12	8.20
	53	<1.00	8.20	8.85
	52	<1.00	49.45	8.25
	49	<1.00	26.02	8.50
	55	2.63	20.37	8.75
	56	2.97	38.52	8.70
	56	2.36	29.39	8.65

continued

Table A 7(i)(continued)

	28	<1.00	39.50	8.70
	28-31	<1.00	30.31	8.25
	31	3.63	22.30	8.25
	30-31	<1.00	28.69	9.05
	30-32	2.95	28.32	7.85
	32	<1.00	NA	6.75
	28	8.04	34.12	8.05
3	NA	7.88	25.90	6.46
	3	6.36	14.85	6.12
	NA	3.36	30.08	7.98
	NA	1.85	25.10	7.99
	NA	3.95	30.07	7.97
	NA	5.95	30.42	8.02
	NA	6.88	47.90	7.13
	56	4.38	36.69	8.11
	1	3.85	53.38	6.40
	50	2.36	26.87	7.65
	48	2.63*	23.14	7.05
	48-50	3.36	24.30	7.66
	45-46	2.63	24.13	7.70
	46	2.59*	22.07	7.40
	19	7.63*	22.29	7.05
	35	2.36	28.47	7.65
	>56	2.36	28.40	9.10
	>56	2.36	26.40	8.80
	>56	5.18	29.28	9.05
4	1	1.36	34.90	5.80
	7	1.98	19.90	5.90
	14	3.18	20.08	NA
	23	4.32	18.00	7.50
	31	2.18	26.03	8.20
	24	2.97	29.03	8.40
	44	<1.00	21.65	8.50
	51	1.36	19.50	7.90
	56	0.60	20.13	8.10
	63	<1.00	41.27	8.35

*litter contamination by salmonellas in house

Table A 7(ii)

Statistical analysis of data in Table A 7(i)

Period of Time	Correlation Coefficient (R^2) for regression of:-						
	Age v Salmonellas	Age v MC%	Age v pH	pH v MC%	pH v Salmonellas	MC v Salmonellas	Age: pH:MC
0-15	45.0	2.4		2.4		23.8	19.1
0-20	55.4		45.0			54.0	
0-25	63.5		55.4			69.9	
0-30	53.7	0.3	63.5	0.3		68.4	4.3
0-35	53.5		53.7		6.0	57.1	
0-40	40.3	1.1	58.3	1.1	1.5	57.1	12.1
0-45	40.9		53.5				
0-50	40.0		40.3				
0-60			40.9				17.5
15-60	5.90					4.8	
20-60	2.40	0.4		0.4	0.5	3.4	8.5
25-60	1.9		1.9			1.7	
30-60	3.4	0.9	3.4	0.9	5.4	3.2	11.4
40-60	7.8		7.8			0.9	35.4
45-60	20.4		20.4				
10-30	81.1			0.7	18.6	1.9	26.6
10-40					1.5		
20-30			12.1	2.2	0.7		16.2
20-35	0.1	0.2				0.4	
20-40				0.0			
20-60			4.0				
25-35	6.2			0.6	0.8	4.8	55.7
25-40		0.4					
25-60			1.9				

Table A 7(iii)

Mathematical Transformation investigated to relate Moisture Content, pH value and Age of Litter

1. $\frac{\log_{10} pH \times \log_{10} MC}{Age}$

2. $\frac{\log_{10} pH \times MC}{Age}$

3. $\frac{\log_{10} MC \times pH}{Age}$

4. $\log_{10} MC \times \log_{10} pH \times Age$

5. $\log_{10} MC \times pH \times Age$

6. $\frac{MC}{pH}$

7. $\frac{pH}{MC}$

8. $\frac{MC}{pH} \times Age$

9. $\frac{pH}{MC} \times Age$

10. $\frac{1}{pH \times MC \times Age}$

11. $\frac{\log_{10} pH}{\log_{10} MC}$

12. $\log_{10} pH \times \log_{10} MC$

13. $\log_{10} pH \times \log_{10}$

14. $\log_{10}$ MC x $\log_{10}$ Age

15. $\frac{\log_{10} \text{MC}}{\text{pH}}$

16. $\frac{\log_{10} \text{pH}}{\text{MC}}$

17. $(\text{pH})^2 \times (\text{MC})^2$

18. $(\text{pH})^2 \times \text{Age}$

19. $(\text{pH})^2 \times \text{MC}$

20. $(\text{pH})^2 \times \text{Age}$

21. $(\text{MC})^2 \times \text{pH}$

22. $(\text{MC})^2 \times \text{Age}$

23. $(\text{Age})^2 \times \text{pH}$

24. $(\text{Age})^2 \times \text{MC}$

25. Age x MC

26. Age x MC x pH

27. MC x pH

28. MC x Age

29. MC x Age x pH

30. pH x MC

31. pH x Age

32. pH x Age x MC

33. pH x Age x MC

Table A 7(iv)

The persistence of Salmonella typhimurium in litter at various sites within broiler houses under commercial conditions

(a) Total area of house

House No.	Site No.	Moisture Content	pH	No. of Salmonellas/g
1	1	20.7	7.4	1
	2	28.33	8.1	<1
	3	23.19	7.2	<1
	4	54.99	7.5	<1
	5	34.91	7.2	<1
	6	31.58	7.2	<1
	7	66.7	7.4	<1
	8	20.0	7.6	<1
	9	16.7	7.1	<1
	10	NA	7.1	<1
	11	31.15	7.2	<1
	12	NA	7.1	<1
	13	18.1	7.8	<1
	14	16.0	7.6	<1
	15	52.68	7.1	<1
	16	28.31	7.4	<1
	17	13.7	7.5	<1
	18	33.63	6.6	<1
	19	4.5	7.1	<1
	20	33.4	6.6	<1
	21	41.0	6.9	<1
	22	46.87	7.3	<1
	23	17.72	7.6	<1
	24	33.68	7.3	<1
	25	46.7	7.4	<1
2	1	32.1	7.1	<1
	2	61.54	7.3	<1
	3	16.31	7.2	<1
	4	66.0	7.4	<1
	5	44.32	7.6	<1
	6	19.24	7.0	<1
	7	30.0	7.0	<1
	8	23.8	7.6	<1
	9	25.00	7.9	<1
	10	21.20	7.7	<1
	11	31.20	7.1	<1
	12	22.08	7.3	<1
	13	18.07	7.2	<1
	14	45.84	7.5	<1
	15	21.7	7.5	<1
	16	36.00	7.4	<1
	17	21.46	6.8	<1
	18	23.28	7.0	<1
	19	42.98	6.9	<1
	20	35.39	7.7	<1
	21	21.43	7.8	<1
	22	20.00	7.0	<1
	23	29.47	7.1	<1
	24	29.09	7.3	<1
	25	21.42	7.2	<1
3	1	59.00	6.9	<1
	2	51.13	7.3	<1
	3	82.28	7.3	<1
	4	66.32	7.2	1.95
	5	53.3	7.6	<1
	6	79.31	7.4	<1
	7	71.67	8.1	<1
	8	76.81	7.2	<1
	9	45.01	7.5	<1
	10	65.09	7.2	<1
	11	68.42	7.2	<1
	12	33.3	7.4	<1
	13	80.00	7.6	<1
	14	83.70	7.1	<1
	15	NA	7.1	<1
	16	68.85	7.2	<1
	17	NA	7.1	<1
	18	81.9	7.8	<1
	19	84.00	7.6	<1
	20	47.32	7.1	<1
	21	71.69	7.4	<1
	22	86.3	7.5	<1
	23	82.28	6.6	<1
	24	55.00	7.1	<1
	25	66.70	6.6	<1

Table A 7(iv) [continued]

4	1	54.00	7.1	1.06
	2	NA	7.8	present in 1g
	3	NA	7.6	present in 1g
	4	18.0	8.2	present in 1g
	5	48.90	8.0	<1
	6	81.52	8.0	<1
	7	50.20	8.4	<1
	8	NA	8.5	<1
	9	63.00	8.2	present in 1g
	10	64.00	7.7	<1
	11	45.11	8.0	3.36
	12	75.06	8.2	present in 1g
	13	58.22	8.11	<1
	14	60.00	7.5	1.30
	15	66.00	7.4	present in 1g
	16	84.40	7.4	1.36
	17	80.00	8.3	present in 1g
	18	87.00	8.4	<1
	19	68.79	8.1	<1
	20	89.00	8.4	<1
	21	69.00	8.6	2.06
	22	NA	7.5	present in 1g
	23	44.70	8.1	present in 1g
	24	NA	7.4	<1
	25	74.72	7.8	present in 1g

Table A 7(iv) [continued]

(b) Transect along length of house

House No.	Site No.	Features	Moisture Content %	pH	No. of Salmonellas
1 A	1		NA	6.80	<1.00
	2		20.00	7.80	4.60
	3	Drinker	23.94	6.60	<1.00
	4	Trough	17.06	6.80	<1.00
	5		2.86	7.70	<1.00
	6	Drinker	20.00	8.06	<1.00
	7	Trough	18.35	7.40	<1.00
	8		6.20	8.00	<1.00
	9		13.19	8.60	<1.00
	10		7.55	8.40	<1.00
	11		39.29	8.60	<1.00
	12		29.70	8.40	<1.00
	13	Trough	16.50	7.80	<1.00
	14	Drinker	26.19	8.20	<1.00
	15		23.86	7.90	<1.00
	16	Trough	39.51	6.65	<1.00
	17		6.16	6.60	<1.00
	18		23.38	7.10	3.32
	19	Wall	25.81	7.60	<1.00
C	1	Door	24.56	7.40	<1.00
	2		41	7.20	NA
	3	Space	38.9	7.00	<1.00
	4		NA	NA	NA
	5		NA	NA	1.36
	6		17.86	6.80	<1.00
	7	Trough	18.07	6.80	<1.00
	8		24.69	7.80	<1.00
	9	Drinker	32.00	8.60	<1.00
	10		28.32	7.80	<1.00
	11	Drinker	27.10	8.10	<1.00
	12		24.55	8.20	<1.00
	13	Trough	12.81	6.10	<1.00
	14	Drinker	40.00	8.20	<1.00
	15		20.00	7.6	<1.00
	16		12.99	6.70	2.62
	17		29.63	6.60	<1.00
	18		20.25	7.60	2.63
3 A	1	Wall	37.20	6.7	<1.00
	2		51.00	8.2	<1.00
	3	Drinker	84.13	NA	<1.00
	4		58.00	7.5	present in 1g
	5	Trough	33.40	7.9	present in 1g
	6		79.92	8.2	<1.00
	7		sample lost		
	8		sample lost		
	9	Drinker	63.10	6.2	present in 1g
	10	Trough	36.84	7.2	present in 1g
	11		70.00	7.4	3.42
	12		53.41	8.3	present in 1g
	13		65.00	8.4	<1.00
	14	Drinker	NA	5.2	present in 1g
	15		NA	8.4	0.36
	16	Trough	NA	7.8	<1.00
	17	Drinker	72.10	4.9	<1.00
	18		62.68	8.1	<1.00
	19		68.67	7.6	1.11
	20	Drinker	67.37	8.2	<1.00
	21	Wall	58.09	7.5	present in 1g
1 B	1		19.05	NA	<1.00
	2		17.80	7.4	<1.00
	3	Heater	5.80	6.6	<1.00
	4		20.26	6.9	<1.00
	5	Heater	10.60	6.6	<1.00
	6		23.08	7.8	<1.00
	7	Heater	7.23	6.6	<1.00
	8		10.70	6.6	<1.00
	9	Heater	9.56	6.6	1.66
	10		3.00	6.6	2.66
	11	Heater	12.00	7.7	<1.00
	12		13.42	7.7	<1.00
	13	Heater	15.00	6.0	<1.00
	14		18.30	7.8	<1.00
	15	Heater	16.75	7.1	<1.00
	16		17.92	7.3	1,95
	17	Heater	21.10	7.3	<1.00
	18		23.00	8.1	<1.00
	19	Heater	12.00	6.9	<1.00
	20		27.38	8.0	<1.00
	21	Heater	33.62	6.6	<1.00

2	B	1		16.00	6.6	<1.00
		2		19.88	6.9	<1.00
		3	Heater	15.50	7.0	<1.00
		4		18.90	6.8	<1.00
		5	Heater	9.06	7.4	<1.00
		6		21.50	7.2	<1.00
		7	Heater	14.40	6.9	<1.00
		8		32.30	7.1	<1.00
		9	Heater	9.58	7.3	<1.00
		10		19.15	7.2	<1.00
		11	Heater	18.11	7.0	<1.00
		12		43.00	7.2	<1.00
		13	Heater	32.00	6.8	<1.00
		14		27.80	7.0	<1.00
		15	Heater	33.01	7.1	<1.00
		16		20.84	7.3	<1.00
		17	Heater	21.21	6.7	<1.00
		18		22.00	7.0	<1.00
		19	Heater	8.00	7.2	<1.00
		20		75.80	7.4	<1.00
3	B	1		71.28	8.4	0.7
		2	Heater	45.62	7.9	present in 1g
		3		72.00	7.9	<1.00
		4	Heater	56.55	8.1	present in 1g
		5		NA	8.2	present in 1g
		6	Heater	69.23	7.8	<1.00
		7		NA	8.3	1.48
		8	Heater	60.00	7.9	<1.00
		9		NA	8.3	<1.00
		10	Heater	NA	8.0	present in 1g
		11		59.65	8.1	present in 1g
		12	Heater	86.65	8.4	2.43
		13		80.00	8.4	present in 1g
		14	Heater	NA	8.2	present in 1g
		15		60.13	7.9	<1.00
		16	Heater	NA	8.1	present in 1g
		17		NA	8.2	2.36
		18	Heater	78.46	8.0	present in 1g
		19		NA	8.3	present in 1g
		20	Heater	56.04	8.2	<1.00

Table A 7(v)

The Persistence of Salmonella typhimurium in Litters on the Centre line of Broiler Houses

House No.	Site No.	Feature	Moisture Content	pH	Persistence of Salmonella
A	1	Brooder	19.05	NA	1
	2	Space	17.80	7.4	1
	3	Brooder	5.80	6.6	1
	4	Space	20.26	6.9	1
	5	Brooder	10.6	6.6	1
	6	Space	23.08	7.8	2.60
	7	Brooder	7.23	6.6	1
	8	Space	10.7	6.6	1
	9	Brooder	9.56	6.6	1.66
	10	Space	3.0	6.6	2.66
	11	Brooder	12.0	7.7	1
	12	Space	13.43	7.7	1
	13	Brooder	15.0	6.0	1
	14	Space	18.3	7.8	1
	15	Brooder	16.75	7.11	1
	16	Space	17.92	7.3	1.95
	17	Brooder	21.1	7.3	1
	18	Space	23.0	8.1	1
	19	Brooder	12.0	6.9	1
	20	Space	27.38	8.0	1
	21	Brooder	33.62	6.6	1

Table A 7(iv) [continued]

House No.	Site No.	Feature	Moisture Content	pH	Persistence of Salmonella
B	1	Brooder	6.0	6.6	
	2	Space	19.88	6.9	
	3	Brooder	15.5	7.0	
	4	Space	18.9	6.8	
	5	Brooder	9.06	7.4	
	6	Space	21.5	7.2	
	7	Brooder	14.43	6.9	
	8	Space	22.3	7.1	
	9	Brooder	9.68	7.3	
	10	Space	19.15	7.2	
	11	Brooder	18.11	7.0	
	12	Space	43.0	7.2	
	13	Brooder	22.0	6.8	
	14	Space	27.8	7.0	
	15	Brooder	23.01	7.1	
	16	Space	20.84	7.3	
	17	Brooder	29.81	6.7	
	18	Space	22.0	7.0	
	19	Broiler	8.0	7.2	
	20	Space	35.8	7.4	

Table A 7(v)

Statistical analysis of the Raw Data in Table A 7(iv)

(a) Simple Regressions

	Correlation Coefficient (R^2) for data from house 1	2	3	4	All houses
Site v. MC%	0.60	10.10	3.90	24.40	11.40
Site v. pH	8.60	0.80	8.20	0.70	1.20
Site v. Salmonellas	9.70	0.00	6.50	0.00	0.00
DM v. pH	2.70	1.10	0.60	5.60	7.40
DM v. Salmonellas	6.10	0.30	0.00	0.30	3.90
pH v. Salmonellas	0.00	-	-	2.70	5.40

(b) Mean Values of Data

MC%	68.93 ± 15.1	69.61 ± 11.00		67.9 ± 14.7*
pH	7.99 ± 0.33	7.30 ± 0.29		7.29 ± 0.33
Salmonellas/g	0.052± 0.20	0.00		0.68 ± 0.39

Table A 8(i)

The Survival of Salmonellas at 48h in Sawdust with various levels of Formaldehyde

% Treated Sawdust	Formaldehyde (mg/g)	Persistence of Salmonellas	pH
0	1.8	4.35	6.0
10	1.8	4.30	5.8
20	1.8	4.60	5.7
30	3.4	5.60	5.7
40	11.4	4.05	6.5
50	20	4.05	6.4
60	20	3.00	6.3
70	25	1.30	6.25
80	28	1.30	6.25
90	34	present in 1g	6.2
100	42	<1.00	6.2

Table A 8(ii)

Survival of Salmonella typhimurium in sawdusts exposed in Commercial Poultry House

1. Levels of Fumigation

Site	Sawdust	formaldehyde mg per g at (d) 1	3	5	7
A	Spruce	32	30	16	NT
	Pine	14	2	14	8.6
	Sycamore	20	32	13.4	NT
	Oak	20	2	14.8	NT
	Beech/Oak	18	<1	13.4	12.4
	Larch	34	50	14	NT
	Mixed sawdust from mill	20	16	15	NT
	House sawdust	34	42	13.4	8.4
	House litter	80	NT	8.6	6.2
B	Spruce	66	62	3.8	NT
	Pine	30	46	5.4	NT
	Sycamore	58	50	3.0	2.0
	Oak	64	28	3.8	NT
	Beech/Oak	12	48	9.4	NT
	Larch	66	62	5.6	NT
	Mixed sawdust from mill	62	<1	5.2	NT
	House sawdust	62	54	5.6	NT
	House litter	26	NT	4.4	0.6
C	Spruce	20	16	2.0	0.4
	Pine	30	20	5.4	5.8
	Sycamore	26	20	2.4	0.4
	Oak	30	10	0.6	0.4
	Beech/Oak	90	26	10.6	14.0
	Larch	10	4	2.6	0.4
	Mixed sawdust from mill	24	6	3.0	0.4
	House sawdust	32	20	1.8	1.2
	House litter	8	nt	0.4	0.2

Table A 8(iii)

Sampling Station	Sawdust	Number of Salmonellas isolated at 1	3	5	7
A	Spruce	<1.00	<1.00	<1.00	<1.00
	Pine	<1.00	<1.00	<1.00	<1.00
	Sycamore	<1.00	<1.00	<1.00	<1.00
	Oak	<1.00	<1.00	<1.00	<1.00
	Beech/Oak	<1.00	<1.00	<1.00	<1.00
	Larch	<1.00	<1.00	<1.00	<1.00
	Mixed sawdust from mill	<1.00	<1.00	<1.00	<1.00
	House litter	<1.00	<1.00	<1.00	<1.00
B	Spruce	<1.00	<1.00	<1.00	<1.00
	Pine	<1.00	<1.00	<1.00	<1.00
	Sycamore	<1.00	<1.00	<1.00	<1.00
	Oak	<1.00	<1.00	<1.00	NT
	Beech/Oak	<1.00	<1.00	<1.00	<1.00
	Larch	<1.00	<1.00	<1.00	NT
	Mixed sawdust from mill	<1.00	<1.00	<1.00	NT
	House sawdust	<1.00	<1.00	<1.00	NT
	House litter	<1.00	<1.00	<1.00	NT
C	Spruce	<1.00	<1.00	1.00	1.00
	Pine	<1.00	4.38	6.38	1.00
	Sycamore	<1.00	3.04	3.38	1.00
	Oak	<1.00	<.63	1.88	1.00
	Beech/Oak	<1.00	<1.00	<1.00	<1.00
	Larch	<1.00	<1.00	<1.00	<1.00
	Mixed sawdust from mill	<1.00	<1.00	<1.00	<1.00
	House sawdust	<1.00	<1.00	<1.00	<1.00
	House litter	3.38	5.38	6.38	NT

Inoculum 1 d - 4.63/g
3 d - 5.53/g
5 d - 4.53/g
7 d - 4.28/g

Table A 9(i)

(a) Chemical Analysis of Poultry Litter as cited in literature (% DM)

Parameter \ Authors(s)	Bhattach & Fontenot (1966)	Canni (1969)	Porigs-Bims (1969)	Szelengi (1971)	Rossi (1972)	Harmon et al (1974)	Harmon et al (1975)	Collison et al (1976)	Caswell et al (1977)	Caswell et al (1978)	Meisner & Vibo (1978)	Patkowsk & Sokola (1980)
Crude protein	30.6	30.8	28.8	26.4	26.1	34.70-38.89	26.75	22.5	73.3	31.5-40.0	31.81	17.0
True protein												
Ammonical nitrogen												
Soluble nitrogen												
Non-protein nitrogen												
N-free extract	33.1	29.8	46.0	33.4	13.7	26.34-32.32	32.04	26.6	37.3	24.7-26.1	32.68	
Crude fat		2.7	1.5	2.2	1.5	4.5-5.59	1.53	1.0	2.8	2.4-3.5	3.21	
Ash	19.0	13.37	15.9	17.0	18.4	11.64-11.69	16.71	22.3	13.3	13.8-19.4	11.83	
Water soluble carbohydrate										2.6-3.6		
Crude fibre	14.6	11.6	7.5	13.2	40.3	16.84-17.49	23.0	27.6	19.3	19.2-19.5	11.62	
Detergent fibre												
Total phosphate	2.26		2.39			1.26					1.44	1.1
LGMN	10.4											

Table A 9(i)

(b) Nitrogen Compounds in Poultry Litter as cited in literature (% DM)

Author	Bhattachang & Fontenot (1966)	Messer (1971)	Shannon et al (1973)	Salter & Fulford (1974)	Caswell (1975)	Harmon (1975)	McNabb & McNabb (1975)	Caswell (1978)
Total nitrogen	44.38		11.8		4.04			5.0-6.8
Soluble nitrogen								
Soluble non-protein N								
Uric nitrogen	28.80	0.2-97.8	6.9		4.12		55-72%	26-34.6
Urea nitrogen	2.81			118-306	0.85-0.88		2-11%	
Ammonia	15.4				0.38-0.39	0.91	11-21%	12.9-15.0
Creotine nitrogen	3.64							
Units		mg/g		mg/g				% total N

Table A 9(i)

(c) Levels of Amino acids in Poultry Litter as cited in literature

Amino acid	Authors Bhattacharga & Fonterot (1966)	Parigi-Bin (1966)	Wilkie & van der Merwe (1974)
Lysine	0.630	2.2	0.622
Proline	0.918	3.2	0.848
Aspartic acid	1.119	4.2	1.234
Threonine	0.561	2.2	0.624
Serine	0.567	2.4	0.729
Glutamine	2.2	6.3	2.156
Glycine	2.116	5.5	0.985
Alanine	0.873	3.3	0.940
Valine	0.818	2.4	0.726
Cysteine		1.4	
Methionine	0.127	0.9	0.287
Isoleucine	0.633	1.7	0.537
Leucine	0.994	3.4	1.107
Tyrosine	0.324	1.4	0.462
Phenylalanine	0.553	1.9	0.626
Histidine	0.239	1.1	0.329
Arginine	0.501	2.3	0.677
Ornithine		0.4	
Unit			
Basal material	Wood		Wood

Table A 9(i)

(d) Minerals in Litter as Cited in Literature

	Conn et al. (1969)	Morrison (1969)	Webb (1971)	Shannon et al. (1973)	Wilkie & van der Merwe (1972)	Kneale & Garstang (1975)	Labosky (1977)	Meisner & Uibe (1978)	Patkowska & Sakolo (1980)	Doear et al. (1981)
Al					295.6					
A		15.30ppm	1.1-59.7			1.4				
Cc					3.43			2.25		
Cd										
Co					29.5	0.36	1.7-22		2.5	11.2-102.7
Cn			37.99	7.3	711.9				10.8	
Fe				0.23	1.18					
K				1.44	0.44				1.1	
Hg	0.54			0.78	336.1				10	
Mn				33.4					1.5	
Mo									0.38	
Na				3.7						
Po					1.51			1.44	2.7	
Se										
Si										
Zn				35	243.0				25	
Br				1.9						
Cl				1.08		0.6				
I_2						4.4				
Units	%DM	ppm	ppm	ppm	ppm	ppm	-	-	mg/100g	mg/g

Table A 9(i)

(e) Chemicals in Litter Originating from Medication as cited in literature (ppm unles stated)

Chemical \ Author(s)	Morrison (1969)	Messer (1971)	Webb & Fortenot (1975)	Calvert et al. (1978)
Amprolicium			0-77.0	
Arsenic	15-30	0.1-76.6mg/g		
Chlortetracycline			0.8-26.3	
Estrone				present
Furazolidone		3.3-25.1		
Neomycin			0	
Nicarbozin			35.1-15.2	
Nitrofurazone		4.5-39.0		
Oxytetracycline			5.5-29.1	
Penicillin			0-25 unit/g	
Testosterone				9.36mg/g
Zinc bacitracin			0.8-36 unit/g	

Table A 9(ii)

The Relationship between Moisture Content and Water Activity of a Mature Litter

Relative Humidity	Dry Matter	Mean Moisture Content
100	42.1)	
	58.07)	
	60.15)	47.05
	51.09)	
88.5	87.22)	
	86.80)	
	87.25)	13.03
	86.62)	
56.8	90.13)	
	90.63)	
	90.42)	9.50
	90.82)	
36.8	93.03)	
	92.95)	
	93.10)	6.93
	93.21)	
18.8	94.42)	
	94.52)	
	94.71	5.45
	ND)	
1	96.40)	
	96.88)	
	97.40)	3.04
	ND)	

Table A 9(iii)

Inhibition of Salmonella typhimurium by a range of Volative Fatty Acids

VFA's	Concentration (mg/ml)	Incubation time (h)	at Original pH			
			5		7.2	
			Turbidity	Final pH	Turbidity	Final pH
Formic acid	10	24	5.0	6.0	4.5	6.9
		48	10.0	6.2	11.5	7.2
		96	41.0	7.0	66.0	7.2
	100	24	0	5.0	4.0	6.6
		48	0	5.0	11.0	6.7
		96	0	5.0	31.0	7.2
Acetic Acid	10	24	4.7	5.9	7.7	7.0
		48	12.0	6.4	16.0	7.0
		96	17.0	7.0	49.5	7.8
	30	24	5.5	5.6	5.0	6.7
		48	10.0	6.2	15.0	7.2
		96	41.0	7.0	37.0	7.6
	100	24	0	5.0	2.0	6.5
		48	0	5.4	10.9	7.2
		96	40.0	6.4	35.0	7.3
Propionic Acid	10	24	6.0	5.8	4.5	7.0
		48	10.0	6.4	9.5	7.0
		96	44.0	7.2	33.0	7.7
	30	24	4.2	5.8	0.5	7.6
		48	9.5	6.4	13.0	7.0
		96	54.0	7.2	38.5	7.6
	100	24	0	5.3	3.0	6.65
		48	7.0	5.8	10.5	7.0
		96	60.5	7.2	36.0	7.4
N-Butyric Acid	10	24	4.5	6.0	5.5	7.0
		48	12.0	6.4	16.0	7.2
		96	51.0	7.4	54.0	7.4
	100	24	4.7	6.0	5.2	6.9
		36	9.0	6.4	16.0	7.2
		96	55.0	7.4	76.0	8.0
N-Valeric Acid	10	24	4.0	5.9	1.5	7.0
		36	10.0	6.5	10.5	7.2
		98	41.0	7.2	51.0	7.8
	100	24	1.0	5.7	1.0	7.1
		48	9.0	6.2	11.5	7.2
		48	46.0	7.0	36.0	7.9
Iso-Valeric Acid	10	24	5.0	6.0	5.0	7.0
		48	16.0	6.5	16.0	7.3
		96	52.0	7.0	41.0	7.8
	100	24	2.5	6.0	1.0	7.0
		48	10.0	6.5	10.0	7.2
		96	48.0	7.2	50.5	7.9
Capronic Acid	10	24	5.0	5.9	3.5	6.9
		48	8.0	6.5	11.0	7.2
		96	37.0	7.4	52.0	7.7
	100	24	0.2	5.6	3.0	6.9
		48	5.0	6.0	10.0	7.2
		96	58.5	7.0	57.0	7.7
Control	-	24	6.2	6.0	4.0	7.0
		48	9.5	6.5	9.5	7.2
		96	53.0	7.7	50.0	7.4

Table A 9(iv)

The Persistence of Salmonella typhimurium in Solutions of Various Sodium Salts
(I _ Inhibited; D = Dead)

(a) Number of Salmonellas in solutions containing:-

Medium	Molarity	Ammonium acetate	Ammonium chloride	Ammonium nitrate	Ammonium phosphate	Ammonium orthophosphate	Ammonium sulphate	Ammonium solution
M9	0.001	8.0	7.0	7.0	5.0	8.0	6.0	8.0
	0.005	8.0	6.0	6.0	5.0	9.0	8.0	8.0
	0.01	3.0	6.0	6.0	6.0	9.0	7.0	I
	0.05	3.0	6.0	6.0	3.0	9.0	7.0	D
	0.1	3.0	4.0	6.0	2.0	8.0	7.0	D
	0.2	3.0	3.0	1.0	2.0	6.0	6.0	D
	0.5	I	1.0	I	3.0	3.0	-	ND
	0.75	I	1.0	I	1.0	1.0	-	ND
N	0.001	5.0	5.0	5.0	4.0	5.0	4.0	4.0
Broth	0.005	5.0	4.0	5.0	4.0	4.0	4.0	I
	0.01	5.0	4.0	4.0	5.0	4.0	2.0	D
	0.05	5.0	5.0	4.0	5.0	6.0	4.0	D
	0.1	4.0	5.0	3.0	4.0	6.0	5.0	D
	0.5	I	3.0	I	3.0	1.0	2.0	ND
	0.75	I	2.0	I	3.0	1.0	2.0	ND

(b) pH values of solutions containing:-

Broth	Molarity	Ammonium acetate	Ammonium chloride	Ammonium nitrate	Ammonium phosphate	Ammonium orthophosphate	Ammonium sulphate	Ammonium solution
Mg	0.001	6.6-1.15	6.65-1.2	6.6-0.85	6.5-1.1	6.65-0.85	6.6-1.15	6.9-1.0
	0.005	6.6-0.9	6.6-1.5	6.6-1.5	6.5-1.05	6.75-0.7	6.6-1.3	8.05-2.25
	0.01	6.6-0.65	6.6-1.3	6.6-0.3	6.5-1.05	6.85-0.95	6.6-1.15	9.2-0.95
	0.05	6.6+0.25	6.6-1.3	65.-0.85	6.1-0.8	7.1	6.6-1.3	10.2
	0.1	6.6+0.05	6.5-1.1	6.4-0.75	5.9-0.65	7.4	6.5-1.1	10.5
	0.2	6.5-0.5	6.0-0.7	6.2-0.1	5.1-0.1	7.6	6.5-1.2	10.9
	0.5	6.5+0.5	6.1-0.2	6.4-0.2	5.15	7.6+0.1	ND	ND
	0.75	6.5	6.05+0.05	6.2	4.8	7.0+0.2	ND	ND
N	0.001	7.2+0.2	7.3+0.2	7.3+0.3	6.9+0.3	7.05+0.6	6.91-	9.0-0.3
Broth	0.005	7.3+0.1	7.4	7.35-0.25	6.6-0.15	7.5+0.15	7.3+0.2	9.7-0.3
	0.01	7.4-0.1	7.4	7.35-0.3	6.25+0.4	7.6+0.1	7.31+0.15	10.0-0.1
	0.05	7.4-0.2	7.3+0.2	7.2-0.3	5.65+0.15	7.7+0.05	7.3+0.15	10.7-0.7
	0.1	7.0+0.2	7.2	7.1-0.35	5.4+0.5	7.75	7.2+0.2	11.0-0.2
	0.2	6.8+0.3	7.0-0.1	6.9-0.2	5.2	7.7-0.05	7.1+0.1	11.25
	0.5	6.8	7.0-0.05	6.9+0.55	4.95-0.05	7.9-0.05	7.1	ND
	0.71	6.8	6.8	6.85+0.15	4.8	7.95+0.05	7.05-0.25	ND

*pH at 0 time I difference in pH reading at 48h

(c) Level of Ammonia ions as determined with Nessler Reagent

Borth	Molarity	Ammonium acetate	Ammonium chloride	Ammonium nitrate	Ammonium phosphate	Ammonium orthophosphate	Ammonium sulphate	Ammonium soluiotn
ND	0.01	+	+	+	+	++	+	+++
	0.05	++	++	++	++	++	++	+++
	0.1	++	++	++	++	++	++	++++
	0.5	+++	+++	+++	++	+++	+++	++++
	0.1	+++	+++	+++	+++	+++	+++	++++
		+++	+++	+++	+++	+++	+++	++++
		+++	+++	+++	+++	+++	+++	++++
	0.75M	+++	++++	+++	++++	+++	white	++++

Colour change 6hr Nessler Reagent added*
*Code in Section 2

Table A 9(vi)

The Inter-relationship between Water Activity and Temperature in Poultry Litter (MC% original = 34.4%)

Water Activity Units	Storage Temperature	Number of Salmonellas persisting 1/g
0.1	4	3.004
	13	3.004
	26	3.004
	37	0.43
0.11-0.15	4	2.97
	13	2.97
	26	2.63
	37	4.004
0.35	4	3.18
	13	3.38
	26	1.38
	37	<1.00
0.42-0.44	4	<1.00
	13	lost
	26	0.60
	37	0.60
0.63-0.66	4	4.004
	13	0.004
	26	lost
	37	4.004
0.84-0.86	4	4.004
	13	4.004
	26	4.004
	37	4.004
1.0	4	>4.004
	13	>4.004
	26	>4.004
	37	>4.004

Table A 9(v)

The Interaction of Water Activity and pH on the Growth of Salmonella typhimurium at two temperatures

(A) Growth in Nutrient Broth with Water Activity adjusted with sodium chloride

1 At 26°C in shaken culture

(a) Persistence of Salmonellas (see footnote for interpretation of symbols in parenthesis)

pH of original broth	Napholometer reading (units) in solution with Aw=					
	Control	0.98	0.96	0.95	0.94	0.70
Control	84	34	2	0	0	0
8	58	36	2	0	0	0
8.5	66	36	3.5	0	0	0
9	72	4.5	3.5	0	0	0
9.5	55	2.5	0	0	0	0
10	51	2	0	0	0	0

(b) Initial pH of broth (Final pH in parenthesis)

pH of original broth	pH value in solution with Aw=					
	Control	0.98	0.96	0.95	0.94	0.70
Control	7.9	7.9(7.25)	7.7(7.4)	7.65(6.75)	7.6(7.05)	7.6(7.3)
8	8.3(7.1)	8.0(6.4)	8.2(6.8)	8.2(7.3)	8.25(6.8)	8.3(7.8)
8.5	8.8(7.4)	8.45(6.75)	8.55(7.8)	8.6(7.8)	8.6(8.4)	8.6(8.0)
9	9.0(NT)	8.85(7.3)	8.85(8.2)	8.9(7.1)	8.9(8.1)	8.85(8.4)
9.5	9.6(7.65)	9.3(8.7)	9.3(8.85)	9.25(8.9)	9.3(8.95)	8.3(9.0)
10	9.9(8.65)	9.5(8.8)	9.9(9.0)	9.25(9.4)	9.55(8.7)	9.6(9.1)

Table A 9(c) [continued]

2 At 26°c in static culture

(a) Persistence of Salmonellas (see footnote for interpretation of symbols in parenthesis)

pH of original broth	Napholemeter reading (units) in solution with Aw= Control	0.98	0.96	0.95	0.94	0.70
Control	49	15	13	2	0 (++-)	0 (+--)
8	47	11	0 (+++)	0 (+++)	0 (+++)	0 (+--)
8.5	46	8	0 (++-)	0 (++-)	0 (---)	0 (+--)
9	33	2	0 (+--)	0 (++-)	0 (+--)	0 (+--)
9.5	7	0 (+--)	0 (+--)	0 (+--)	0 (+--)	0 (+--)
10	0 (++-)	0 (++-)	0 (+--)	0 (+--)	0 (+--)	0 (+--)

(b) pH of Initial Solution (Final pH in parenthesis)

pH of original broth	pH value of solution with Aw= Control	0.98	0.96	0.95	0.94	0.70
Control	7.9(7.15)	7.9(6.7)	7.7(6.8)	7.65(7.4)	7.6(7.25)	7.6(7.4)
8	8.3(7.0)	8.0(7.05)	8.2(7.8)	8.2(8.05)	8.25(7.8)	8.5(8.0)
8.5	8.8(7.1)	8.45(7.5)	8.55(8.15)	8.6(8.4)	8.6(8.4)	8.6(8.4)
9	9.0(7.4)	8.85(8.25)	8.55(8.5)	8.9(8.4)	8.9(8.7)	8.85(8.7)
9.5	9.6(7.7)	9.3(9.0)	9.3(8.95)	9.25(9.1)	9.3(9.1)	8.3(9.15)
10	9.9(9.5)	9.5(8.3)	9.9(9.45)	9.55(9.4)	9.55(9.4)	9.6(9.2)

Table A 9(v) [continued]

3 At 37°C in static culture

(a) Persistence of Salmonellas (see footnote for interpretation of symbols in parenthesis)

pH of original broth	Napholometer reading (units) in solution with Aw= Control	0.98	0.96	0.95	0.94	0.70
Control	84	6	4	2	0 (+-)	0 (+--)
8	57	14	0 (++)	12	0 (++)	0 (+--)
8.5	56	6	0 (+-)	0 (+--)	0 (+--)	0 (+--)
9	59	6	0 (++)	0 (++)	0 (+--)	0 (+--)
9.5	42	0 (++)	0 (+--)	0 (+--)	0 (---)	0 (---)
10	0 (++)	0 (+-)	0 (---)	0 (---)	0 (---)	0 (---)

(b) pH value of Initial Solution (Final pH in parenthesis)

pH of original broth	pH value of solution with Aw= Control	0.98	0.96	0.95	0.94	0.70
Control	7.2(7.0)	7.9(6.6)	7.7(6.6)	7.65(7.9)	7.6(7.0)	7.6(7.3)
8	8.3(7.0)	8.0(6.8)	8.2(7.25)	8.2(7.6)	8.25(7.4)	8.3(7.9)
8.5	8.8(7.3)	8.45(7.2)	8.55(7.6)	8.6(8.3)	8.6(8.2)	8.6(8.2)
9	9.0(7.3)	8.85(7.2)	8.85(8.15)	8.9(7.2)	8.9(8.5)	8.85(8.4)
9.5	9.6(8.6)	9.3(7.2)	9.3(8.7)	9.25(8.9)	9.3(8.9)	8.3(8.6)
10	9.9(9.1)	9.5(8.9)	9.9(9.2)	9.25(9.1)	9.55(9.2)	9.6(9.2)

Table A 9(v) [continued)

B Growth in Nutrient Broth with Water Activity adjusted with glycerol

1 At 26°C or 37°C in static culture

(a) Persistence of Salmonellas

	Napholemeter reading (units) in solution with Aw at (°C)			
	0.95		0.90	
pH	26°C	37°c	26°C	37°c
9	10	3	5	2.5
9.5	5	0 (---)	8	0 (+++)
10	0 (+++)	0 (---)	0 (---)	0 (---)

(b) pH level of Initial Broth (Final pH in parenthesis)

	pH value in solution with Aw at (°C)			
	0.95		0.90	
pH	26°C	37°C	26°C	37°C
9	9.0(8.35)	9.0(7.2)	9.2(7.8)	9.2(7.2)
9.5	9.3(7.55)	9.3(6.9)	9.5(8.25)	9.5(7.1)
10	9.7(9.1)	9.7(8.4)	9.7(9.6)	9.7(8.4)

Table A 9(b) [continued]

Growth in Nutrient Broth with Water Activity adjusted with Glycerol as agent over range Aw = 0.99 to Aw = 0.65

(a) At 26°C in shaken culture (see footnote for interpretation of symbols in parenthesis)

pH of original solution	Napholometer reading (unit) of solution with Aw = Control	0.95	0.90	0.85	0.75	0.65
Control	80	NT	NT	NT	NT	
8	NT	24	14	6	2	2
8.6	NT	25	25	15	2	0
9	NT	23	15	9.9	5	6
9.6	NT	25	24	4.5	4.5	5

(b) pH value of initial solution (Final pH in parenthesis)

pH of original solution	pH value of solution with Aw = Control	0.95	0.90	0.85	0.75	0.65
Control	-(6.8)	NT	NT	NT	NT	NT
8	NT	8.0(6.8)	7.8(6.5)	7.9(7.4)	7.8(7.6)	7.8(7.8)
8.5	NT	8.1(7.3)	8.05(7.0)	8.1(7)	8.05(8.0)	8.2(8.0)
9	NT	8.35(7.2)	8.4(7.4)	8.3(7.3)	8.3(8.2)	8.4(8.2)
9.5	NT	8.7(8.0)	8.7(7.1)	8.7(8.0)	8.7(8.3)	8.7(8.9)

Table A 9(w) [continued]

C Growth in M_9 Broth with Water Activity adjusted with sodium chloride

1 At 26°C in static culture

(a) Persistence of Salmonellas (see footnote for interpretation of symbols in parenthesis)

pH of original broth	Napholometer reading (unit) of solution with Aw = Control	0.98	0.96	0.95	0.94
Control	4	1.5	0 (+++)	0 (++-)	0 (++-)
8	2	0 (+++)	0 (++-)	0 (+--)	0 (---)
8.5	2	0 (+--)	0 (+--)	0 (---)	0 (---)
9	0 (+++)	0 (---)	0 (---)	0 (---)	0 (---)

(b) pH vlaue of Initial Solution (Final pH in parenthesis)

pH of original broth	Control	pH value of solution with Aw = 0.98	0.96	0.95	0.94
Control	6.35(6.2)	6.3(6.15)	6.0(6.1)	5.95(6.1)	5.3(5.5)
9	7.8(6.8)	8.1(7.6)	8.1(7.8)	8.1(7.8)	8.0(7.7)
9.5	8.8(8.3)	8.8(8.3)	8.8(8.3)	8.75(8.3)	8.65(8.2)
10	8.85(8.3)	8.8(8.4)	8.8(8.35)	8.7(8.3)	8.7(8.3)

Table A 9(a) [continued]

2 At 26°c in shaken culture

(a) Persistence of Salmonellas (see footnote for interpreation of symbols in parenthesis)

pH of original broth	Napholometer reading (unit) of solution with Aw = Control	0.98	0.96	0.94	0.70
Control	22	0	0	0	0
8	29.9	0	0	0	0
8.5	0	0	0	0	0
9	0	0	0	0	0

(b) pH value of Initial Solution (Final pH in parenthesis)

pH of original broth	pH value of solutions with Aw= Control	0.98	0.96	0.94	0.70
Control	6.35(6.0)	6.3(6.2)	6.0(-)	8.95(5.5)	8.3(-)
8	7.8(6.8)	8.1(7.7)	8.1(7.8)	8.1(7.8)	8.0(7.6)
8.5	8.8(8.2)	8.8(-)	8.8(8.2)	8.75(8.05)	8.65(7.5)
9	8.85(7.9)	8.8(7.2)	8.8(8.1)	8.7(8.1)	8.7(8.05)

Table A 9(p) [continued]

D Growth in M_9 broth with Water Activity adjusted with glycerol

1 At 26°C in static culture

(a) Persistence of Salmonellas (see footnote for interpretation of symbols in parenthesis)

pH of original broth	Naphelometer reading (unit) of solution with Aw = Control	0.98	0.90	0.85	0.75	0.65
Control	7.2	NT	NT	NT	NT	NT
8	NT	11	5	3	2 (+++)	1 (---)
8.6	NT	11	5	4	0* (+++)	0 (+++)
9	NT	11	7	5	0* (+++)	0 (---)
9.6	NT	14	7	4	0* (+--)	0 (---)

(b) pH value of Initial Solution

pH of original broth	pH value of solution with Aw = Control	0.95	0.90	0.85	0.75	0.65
Control	6.8	NT	NT	NT	NT	NT
8	NT	8.0	7.8	7.9	7.8	7.8
8.6	NT	8.1	8.05	8.1	8.05	8.2
9	NT	8.35	8.4	8.3	8.3	8.4
9.6	NT	8.7	8.7	8.7	8.7	8.7

Table A 9(v) [continued]

3 At 37°C in static culture

(a) Persistence of Salmonella (see footnote for interpretation of symbols in parenthesis)

pH of original broth	Naphelometer reading (unit) of solution with Aw = Control	0.98	0.96	0.94	0.70
Control	7	0* (+++)	0* (+++)	0 (---)	0 (---)
8	2.3	0* (+++)	0* (+++)	0 (++-)	0 (---)
8.5	0 (+--)	0 (---)	0 (---)	0 (---)	0 (---)
9	0 (---)	0 (---)	0 (---)	0 (---)	0 (---)

(b) pH vlaue of Initial Solution (Final pH in parenthesis)

PH of orginal broth	Control	pH value of solutions with Aw = 0.98	0.96	0.94	0.70
Control	6.35(5.95)	6.3(6)	6.0(6)	5.95(5.9)	6.3(5.45)
8	7.8(5.6)	8.1(7.2)	8.1(7.6)	8.1(7.6)	8.0(7.5=
8.5	8.8(8.0=	8.8(8.0)	8.8(7.95)	8.75(7.95)	8.65(7.8)
9	8.85(8.0)	8.8(7.9)	8.8(7.9)	8.7(7.9)	8.7(7.9)

Footnote

+++ - growth undiluted

-++ - growth 10^{-1} dilution

--+ - growth 10^{-2} dilution

--- - no growth 10^{-3} dilution

* - GROWTH ON BS AGAR

Table A 10(i)

Bacteriological Analysis of Litter of Various Ages from Two Enterprises

Enterprise	Age of Litter	MC (%)	pH	Bacterial count/g or:- Total Aerobic Count N Agar	PC Agar +1% litter extract	Anaerobic Count	Urea decomposing Bacteria	Number of Salmonellas	Ammonia level (mg/g)
A	3	50	6.0	8.26	8.51	8.20	7.56	3.95	NT
	6	16.5	6.6	10.13	9.80	9.44	8.97	4.85	NT
	16	13.9	6.55	10.12	10.16	9.32	7.28	4.63	NT
	21	28.5	6.8	10.13	10.09	8.99	8.66	2.63	NT
	35	24.1	7.4	10.18	10.19	8.21	8.66	0.60	NT
	42	30.0	7.6	10.09	9.90	7.95	7.56	0.95	NT
B	2-4	13.8	6.8	7.91	NA	7.78	8.97	4.48	0.002
	7	61.5	6.4	9.64	9.18	9.18	8.97	4.46	0.021
	14	22.7	7.6	10.67	10.30	8.39	8.97	3.32	0.025
	21	20.7	7.1	10.08	11.45	8.50	9.38	3.97	0.027
	28	29.6	8.1	10.30	9.65	7.65	8.97	not present 1g	0.045
	35	24.6	8.1	10.80	8.78	7.48	8.32	not present 1g	0.042
	42	26.9	8.2	8.47	8.57	6.00	6.38	not present 1g	0.051
	49	23.1	7.8	8.91	9.53	.50	7.38	not present 1g	0.054

* Level of inoculum = 3.83/g

Table A 10(ii)

The Microbial flora of Litters from a Commercial House sampled at weekly Intervals

Analysis	Prior to placement		Days after production						
	before fumigation	after fumigation	1	8	15	22	29	36	42
26°C Aerobic Incubation									
HI agar	7.28	6.69	7.32	7.43	8.69	8.45	7.59	NA	8.75
VLhfl agar	NT	7.28	7.48	8.34	8.28	8.52	7.52	NA	8.67
VL agar	NT	NT	NT	7.48	8.43	8.11	7.85	NA	8.75
Urea decomposing agar	NT	NT	NT	<3.00	7.30	7.30	7.78	10.04	10.17
Uric acid decomposing agar	NT	NT	NT	6.39	NT	NT	NT	NA	<3.00
TAT agar	NT	NT	NT	5.78	6.17	NT	6.69	NA	NT
MacConkey agar	NT	NT	NT	5.60	NT	NT	7.53	NA	8.43
Cellulose decomposing agar	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00	NA	<2.00
OAES agar-moulds	1.48	4.58	4.00	5.00	5.60	5.60	NT	NA	4.78
yeasts	>6.00	>6.00	6.00	6.46	6.87	6.87	NT	NA	5.28
Malt agar-moulds	1.90	2.42	3.83	5.30	5.60	5.60	3.85	NA	5.08
yeasts	>6.00	>.500	6.87	6.48	6.92	7.20	5.46	NA	5.43
½TSA agar	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00
C2+ agar	<1.00	<1.00	<1.00	<1.00	4.30	<1.00	<1.00	<1.00	<1.00
26°C Anaerobic Incubation									
HI agar	4.69	4.69	4.00	6.79	8.69	8.58	7.61	NA	6.95
VLhfl agar	5.00	5.00	7.30	7.79	8.11	6.86	NA	7.69	5.90
VL agar	4.69	4.69	7.11	7.34	7.39	6.88	<7.00	NT	6.81
V-K agar	4.00	4.69	6.96	6.62	7.21	6.30	4.60	NT	6.61
TAT agar	<1.00	<1.00	<1.00	5.61	6.26	7.63	NT	NT	6.78
Rogosa agar	<1.00	<1.00	<1.00	<1.00	<1.00	5.30	>5.0	NT	6.51
MacConkey agar	1.69	<1.00	<1.00	0.42	6.61	7.42	NT	NT	6.38
Ethyl violet agar	<1.00	<1.00	<1.00	<1.00	<1.00	5.67	>1.00	NT	<1.00
Urea decomposing agar	<1.00	<1.00	<1.00	<1.00	<1.00	6.91	5.20	NT	<1.00
Uric acid decomponsing agar	<1.00	<1.00	<1.00	1.77	4.38	6.00	4.20	NT	
37°C Aerobic Incubation									
HI agar	<1.00	<1.00	<1.00	5.95	7.59	6.94	6.95	7.77	7.80
VLhfl agar	NT	NT	NT	7.04	7.38	7.39	6.48	7.30	8.36
VL agar	NT	NT	NT	NT	NT	7.93	8.45	8.48	NT
Uric acid decomposong agar	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00
MacConkey agar	NT	NT	NT	NT	NT	7.55	NT	7.44	8.48
37°C Anaerobic Incubation									
HI agar	<1.00	<1.00	<1.00	5.95	7.59	6.94	6.95	7.77	7.80
VLhfl agar	NT	NT	NT	7.04	7.38	7.39	6.48	7.30	8.36
VL agar	NT	NT	NT	6.83	7.20	8.25	7.26	6.48	6.89
TAT agar	<1.00	<1.00	<1.00	NT	NT	5.15	5.92	6.49	6.36
Rogosa agar	<1.00	<1.00	5.11	8.11	6.59	5.85	5.65	5.69	5.32
MacConkey agar	NT	NT	NT	6.42	7.15	6.78	7.55	7.90	5.96
VK agar	NT	NT	NT	NT	NT	4.63	4.63	5.66	6.79
Uric acid decomposing agar	NT	NT	NT	NT	NT	NT	NT	6.00	6.61
55°C Aerobic Incubation									
Cz+ agar	1.00	<1.00	<1.00	<1.00	2.00	<1.00	1.00	1.69	<1.00
½TSA agar	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00
Persistence of Salmonellas									
Inoculum	3.11	3.11	3.11	3.11	3.11	2.32	2.65	2.66	3.30
Number of organisms after 2d	3.83	2.63	2.38	4.38	4.38	7.38	7.77	<1.00	<1.00
% survivors	123.8	84.6	76.5	140.9	140.9	318.1	293.2	0	0
Physical parameters									
pH	7.2	7.2	6.0	5.9	6.0	NT	NT	8.2	8.2
MC %	40.1	28.8	5.3	18.6	23.1	24.2	26.9	24.8	31.8

Table A/6(iii)

The Microbiological Analysis of Litters collected from a Non-commercial poultry enterprise

(a) Total viable count/g

Trial No.	Pen Numbers	Age of birds 0	4	11	22	28	36	49	56
1	1-8	NT	NT	7.33	8.65	8.56	9.30	9.57	NT
1	41-48	NT	NT	6.73	9.44	8.92	9.74	9.59	NT
2	10-17	4.97	5.98	8.47	9.69	9.49	10.32	10.06	10.24
2	32-39	4.64	5.34	7.73	9.46	9.37	9.24	10.11	10.67
(b) Anaerobic Bacteria/g									
1	1-8	NT	NT	7.33	8.37	8.02	8.45	7.2	NT
1	41-48	NT	NT	7.1	8.78	8.10	8.75	7.43	NT
2	10-17	4.34	5.14	7.59	NT	8.04	8.39	9.10	8.62
2	32-39	4.08	5.23	7.96	NT	8.03	6.89	8.47	8.67
(c) Persistence of Salmonella/g									
1	1-8	0.0	5.11	3.59	1.32	1.56	1.63	3.77	3.38
1	41-48	0.0	5.13	2.69	1.66	1.46	2.10	3.37	3.26
Inoculum		3.57	3.52	3.36	3.23	2.32	3.48	3.30	3.40
2	10-17	0.45	2.62	2.38	NT	2.59	2.51	NT	
2	32-39	0.15	3.38	2.29	NT	2.09	2.09	NT	
Inoculum		3.48	4.38	3.36	-	3.08	2.83	-	
(d) Moisture Content									
1	1-8	7.53	26.13	NT	18.39	NT	26.29	36.48	27.89
1	41-48	13.51	31.85	15.18	22.66	NT	36.82	32.16	27.05
		49							
(e) pH value									
1	1-8	NT	6.7	NT	5.7	6.6	6.85	6.8	7.3
1	41-48	NT	6.75	NT	5.8	7.0	6.9	6.7	7.4
2	10-17	5.73	6.47	6.70	6.70	6.35	NT	6.05	NT
2	32-39	5.38	6.18	6.73	6.74	6.38	NT	6.12	NT

Table A11 (i)

The Characteristics of bacteria capable of antagonising Salmonella typhimurium in Laboratory Media isolated from Poultry Litter

(a) Coryneform bacteria test	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. Pigment	cream/ yellow	cream	cream	white	cream	white	cream	cream	white	white	white	cream	cream	cream	cream	white	white
Growth																	
2. aerobic	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3. anaerobic	-	-	-	-	-	-											
4. Motility	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5. Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6. Liquifaction of gelatine	-	±	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7. Liquifaction of nitrates	+	+	+	-	±	-	-	-	-	+	-	-	-	+	-	-	+
8. Fermentation of β-galactase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
9. Fermentation of saccharose	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-
10. Fermentation of L(+) arabinose	+	-	+	-	+	-	-	-	-	+	-	-	-	+	+	-	+
11. Fermentation of mannitol	+	-	+	-	+	-	-	-	-	+	-	-	-	+	+	-	+
12. Fermentation of fructose	+	-	+	-	+	-	-	-	-	+	-	-	±	+	+	-	+
13. Fermentation of glucose	+	-	+	-	+	-	-	-	-	+	-	-	±	+		-	+
14. Fermentation of maltose	+	-	+	-	±	-	-	-	-	+	-	-	±	+		-	+
15. Fermentation of starch	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16. Fermentation of rhamanose	+	-	+	-	+	+	-	-	-	+	-	-	-	+		-	+
17. Fermentation of galactose	+	-	+	-	+	+	-	-	-	+	-	-	-	+		-	+
18. Fermentation of mannose	+	-	+	-	+	-	-	-	-	+	-	-	-	+		-	+
19. Fermentation of sorbitol	+	-	+	-	+	-	-	-	-	+	-	-	-	+		-	+
20. Fermentation of glycerol	+	-	+	-	+	-	-	+	-	+	-	-	-	+		-	+
21. Presence of urea	-	-	±	-	-	-	-	-	-	-	-	+	-	-		-	-
22. Indole test	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
23. Fermentation of H_2S	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	+	+
24. Production of Acetoin	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-
25. Simmon's citrate	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-
26. Cytochrome oxidase	+	+	+	+	+	+	-	-	+	+	+	+	-	+	+	-	-
27. Fermentation of glucose - aerobic	+	-	+	-	+	-											
28. Fermentation of glucose - anaerobic	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	+	+
29. Decomposition of uric acid	+	-	-	-	-	-	+	-	-	+	-	-	-	-	-	+	-
30. Decomposition of sodium hippurate	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	-	-
31. Haemolysis							sl	sl	-	-	-	st					
32. Reaction in Litmus milk	R	NR	AD	NR	A	NR	NR	NR	NR	AR	NR	NR	NR	R	R	NR	NR
33. Reaction in glucose agar	slow	slow	-	slow	-	slow	slow	slow	medium	slow	slow	-	slow	slow	slow	slow	slow
34. Diatase	-	-	slight	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table A 11 (i) [continued]

(b) Other Gram-positive bacteria

Tests	Culture Designation - Micrococcus 1	2	3	4	5	6	7	8	9	10	11	Bacillus sp.
1. Pigment	white	white	orange	cream	white	cream/ yellow	cream	white	white	white	white	cream
2. Growth - aerobic	+	+	+	+	+	+	+	+	+	+	+	+
3. Growth - anaerobic												
4. Motility	-	-	-	-	-	-	-	-	-	-	-	-
5. Catalase	+	+	+	+	+	+	+	+	+	+	+	+
6. Liquifaction of gelatine	+	-	-	-	+	-	-	-	-	-	-	-
7. Liquifaction of nitrate	+	-	+	-	-	+	-	-	-	-	-	-
8. Fermentation of -galactose	-	-	+	-	-	-	-	-	-	-	+	-
9. Fermentation of saccharose	-	+	-	-	+	-	-	-	-	-	-	-
10. Fermentation of L(+) arabinose	-	-	-	+	-	-	-	-	-	-	-	-
11. Fermentation of mannitol	-	+	-	-	-	-	-	-	-	-	-	-
12. Fermentation of fructose	-	+	-	-	+	-	-	-	-	-	-	-
13. Fermentation of glucose	-	+	-	-	+	-	-	-	-	-	-	-
14. Fermentation fo maltose	-	+	-	-	+	-	-	±	-	-	-	-
15. Fermentation of starch	-	-	-	-	-	-	-	±	-	-	-	±
16. Fermentation of rhamanose	-	-	-	-	-	-	-	±	-	-	-	-
17. Fermentation of galactose	-	-	-	-	-	-	-	-	-	-	-	-
18. Fermentation of mannose	-	-	-	-	-	-	-	-	-	-	-	-
19. Fermentation of sorbitol	-	-	-	-	+	-	-	-	-	-	-	-
20. Fermentation of glycerol	-	+	-	-	-	-	-	-	-	-	-	-
22. Indol Test	-	-	-	-	-	-	-	-	-	-	±	+
23. Formation of H_2S	-	±	-	+	-	-	-	-	±	±	-	+
24. Production of Acetoin	-	-	-	-	-	-	-	-	+	+	-	+
25. Simmon's citrate	-	-	-	+	-	+	-	+	-	+	+	±
26. Cytochrome oxidase	+	-	+	+	+	+	-	+	-	-	-	+
27. Fermentation of glucose - aerobic	-	+	-	-	+	-						
28. Fermentation of glucose- anaerobic	-	+	-	-	-	-	-	-	+	+	-	+
29. Decomposition of uric acid	-	-	-	-	-	+	-	-	-	-	-	-
30. Decomposition of sodium hippurate	-	+	-	-	-	-	-	-	+	+	+	+
31. Haemolysis												
32. Reaction in Litmus milk	NR	NR	NR	NR	NR	RAD	NR	NR	R	R	R	NR

Table A 11(ii)

The Antagonism of Salmonella typhimurium by Simple Mixtures of Bacteria derived from Poultry Litter

(pH value in parenthesis, * Nessler reagent 2, ** Nessler reagent 2)

(a) Recombination 1

Culture Identificaiton			
a	I (8.2)		
b	I (8.4)	I (-)	
c	I (8.4)	I (8.4)	I (8.4)
	a	b	c

Culture identification

(b) Recombination 2

Culture Identification			
B	PI (8.3)		
C	PI (8.4)	PI (8.8)	
D	PI (8.5)	PI (8.45)	PI **(8.4)
	A	B	C

Culture Identification

(c) Recombination 3

Culture Identification			
b	PI *(8.4)		
c	PI *(8.3)	PI *(8.4)	
d	I *(8.4)	I *(8.4)	I *(8.4)
	a	b	c

Culture Identification

Table A 11(ii) [continued]

(d) Recombination 4

	Culture	Identification
b	I *(8.4)	
c	I *(8.3)	I *(8.4)
	a	b
	Culture	Identification

(e) Recombination 5

	Culture	Identification
b	PI *(8.4)	
c	I *(8.4)	I *(8.4)
	a	b
	Culture	Identification

(f) Recombination 6

	Culture	Identification
b	PI *(8.4)	
c	PI *(8.4)	PI *(8.4)
	a	b
	Culture	Identificaiton

(g) Recombination 7

	Culture	Identification
b	PI **(8.6)	
c	PI (8.6)	I ***(8.6)
	a	b
	Culture	Identification

Table A 11(ii) [continued]

(h) Recombination 8

Culture
Identification

a PI **(8.5)

c PI (8.6) I ***(8.6)

a b
Culture Identification

Table A 11(iii)

The Persistence of Salmonella typhimurium by Mixed Cultures of Bacteria dervied from Poultry litters assessed by Agar Disc Method

Identification of culture	Plate of Origin 1	2	3	4	5	6	7	8	9
(a) Inhibition of Salmonellas in Agar Disc									
AB	I	PI	PI*	I*	PI*	I*	PI**	PI*	
AC	I	PI	PI*	I*	PI*	I	PI**	I*	
AD	I	PI	I*	-	-	-	-	-	
BC	I	PI	PI*	I*	PI*	I*	I***	I*	
BD	I	PI	I*	-	-	-	-	-	
CD	I	PI**	I*	-	-	-	-	-	
(b) pH of solutions									
AB	8.3	8.3	8.4	8.3	8.4	8.4	8.6	8.4	
AC	8.4	8.4	8.3	8.3	8.4	8.5	8.6	8.4	
AD	8.4	8.5	8.4	-	-	-	-	-	
BC	8.4	8.5	8.4	8.4	8.4	8.4	8.6	8.4*	
BD	8.4	8.45	8.4	-	-	-	-	-	
CD	8.4	8.4	8.4	-	-	-	-	-	

Table A 11(iv)

Antagonism of Simple Mixtures of Bacterial Species to S. typhimurium as detected by Agar Disc Techniques

Culture identification					
b	I*** (8.6)				
c	I* (8.6)	PI* (8.6)			
d	PI* (8.6)	PI* (8.5)	PI* (8.4)		
e	I* (8.6)	PI* (8.3)	I* (8.4)	PI* (8.5)	
f	PI* (8.6)	I* (8.5)	I* (8.6)	I* (8.4)	PI* (8.4)
	a	b	c	d	e

Culture Identification

(pH value shown in parenthesis, Nessler Reagent * code 1

** code 2)

[See Chapter 2 D 6 (i)]

Table A 11(iv)

Inhibition of Salmonella typhimurium by "Supternatant" from Broth Culture and the Effect of Resuscitation in Nutrient Broth (ordered by level of inhibition)

Designation of Culture	Medium*	pH	Inhibition in Supernatant**		After resuscitation**	
			Untreated	Autoclaved	Untreated	Autoclaved
(a) Cultures able to Inhibit Salmonellas in Solution						
Coryneform 1	R	5.1	I	I	I	I
Coryneform 9	R	5.0	I	I	I	I
Coryneform 3	R	5.0	I	I	I	PI
Coryneform 9	T	5.9	I	PI	PI	PI
Coryneform 4	R	5.9	I	PI	I	PI
Bacillus sp.	R	5.9	I	I	PI	+
Coccus 10	B	7.1	PI	PI	PI	PI
Coccus 2	R	5.9	PI	+	PI	+
(b) Culture not able to inhibit Salmonellas in Solution						
Coryneform 5	N	7.6	I	I	I	PI
Coryneform 9	N	7.25	I	I	I	PI
Coccus 6	B	7.05	I	I	I	PI
Coccus 3	N	7.1	I	I	PI	PI
Coccus 9	R	7.1	I	I	PI	PI
Coryneform 1	T	7.1	I	PI	I	PI
Coryneform 1	N	8.2	I	PI	I	PI
Coryneform 2	B	7.4	I	PI	I	PI
Coryneform 2	N	8.2	I	PI	I	PI
Coryneform 7	B	7.4	I	PI	I	PI
Coryneform 11	B	7.1	I	PI	I	PI
Coryneform 13	N	7.4	I	PI	I	PI
Coccus 6	N	7.2	I	PI	I	PI
Coryneform 13	R	7.4	I	PI	I	I
Coryneform 8	B	7.35	I	PI	PI	PI
Coryneform 8	N	8.1	I	PI	PI	PI
Coryneform 10	B	7.1	I	PI	PI	PI
Coccus 9	B	7.2	I	PI	PI	PI
Coryneform 13	T	6.5	I	PI	+	+
Coryneform 9	B	7.5	I	+	I	PI
Coryneform 3	N	7.5	PI	I	PI	I
Coccus 10	N	7.1	PI	PI	PI	PI
Coryneform 4	N	7.1	PI	PI	I	PI
Coryneform 6	T	7.2	PI	PI	I	PI
Coryneform 4	N	7.1	PI	PI	PI	PI
Coryneform 6	T	7.2	PI	PI	PI	PI
Coccus 5	B	7.3	PI	PI	I	PI
Coryneform 5	B	7.1	PI	PI	PI	PI
Coryneform 12	N	7.5	PI	PI	PI	PI
Coccus 1	N	7.2	PI	PI	PI	PI
Coccus 5	N	7.2	PI	PI	PI	PI
Coccus 7	B	7.1	PI	PI	PI	PI
Coccus 7	N	7.6	PI	PI	PI	PI
Coccus 8	N	7.6	PI	PI	PI	PI
Bacillus sp.	B	7.25	PI	PI	PI	PI
Bacillus sp.	T	6.7	PI	PI	PI	PI
Coccus 8	R	6.5	+	PI	PI	PI
Coccus 8	R	6.8	+	+	PI	+

* Medium N - N Broth
B - BPW
R - RCM Broth
T - Tryptocase soya broth

**Inhibition I - Inhibition
PI - Partial inhibition
+ - Growth

Table A 11(v)

Summary of Susceptibility of bacteria antagonistic salmonellas to Antibiotics

Culture	Culture susceptible to:- Streptomycin sulphate	other antibiotics
Coryneform 1	-	BA
Coryneform 2	-	NI, Ba, PG, CP
Coryneform 3	-	IVI, PG, TS
Coryneform 4	-	BA, SM
Coryneform 5	-	BA, PG
Coryneform 6	-	BA, PG
Coryneform 7	-	BA, CP
Coryneform 8	-	BA, PG
Coryneform 9	-	BA, PG
Coryneform 10	-	BA, PG, CP
Coryneform 11	-	PG
Coryenform 12	-	PG
Coryneform 13	-	BA, NE
Coryneform 14	-	BA, PG
Coryneform 15	-	K, NA, NE, PG, PB
Coryneform 16	+	NA, PG, PB
Coryneform 17	-	PG, GM
Coccus 1	-	-
Coccus 2	-	PB
Coccus 3	+	-
Coccus 4	-	AP, BA, PG
Coccus 5	-	PG
Coccus 6	-	Co, NA, NI, T, PA, PG, CP, CM, ITS
Coccus 7	-	BA, PG, CP
Coccus 8	-	BA, PG, SM, TS
Coccus 9	-	Co, K, NA, NE, PG, PB
Coccus 10	-	AB, Co, K, WA, PG, PB, CP, SM
Streptococcus faecium	+	

APPENDIX III. PAPERS SUBMITTED IN SUPPORT OF THIS THESIS

delivery was made the effect of contaminated food on the birds could not be determined. This is a lower degree of food contamination than found by Patterson and Gibbs (1977) who indicated that although in their study the food entering the shed was free from contamination some material became contaminated within the shed. The results from site A showed that this was not the case in this investigation.

Dust collected at 56 d showed the presence of salmonellae in the environment, the dust from the fans giving a better indication of the serotypes than the dust from the brooder hoods. However, *S. montevideo* was not isolated from any dust samples from site A and the source of *S. infantis* in the dust from site B was not found.

The salmonellae in this investigation were introduced into the house by the chicks and quickly became established in the litter and water troughs. These sources then served as foci of infection to infect or re-infect birds at the later stages of rearing. The food did not appear to contribute significantly to the population of salmonellae in the houses.

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TABLE 5

Numbers of birds at slaughter infected with Salmonellae on feet, feathers and in viscera

	Site A		Site B	
	House 1	House 2	House 3	House 4
Feet	0	10% (i)	0	0
Feathers	0	20% (i, a)	0	10% (a)
Liver	0	0	0	0
Heart	0	20% (i)	0	0
Gizzard	0	0	0	0
Intestine	10% (a)	0	0	0

a, *S. agona*; i, *S. infantis*.

DISCUSSION

When the incidences of infection at site A and site B are compared it can be seen that the incidence of infection of the incoming chicks was greater at site A than at site B. However the incidence of salmonellae during the rearing of the birds was greater at site B. This could have been due to a real increase in infection or to improved methodology in the isolation techniques. At the point of slaughter the incidence of infection of birds from site A was greater than that of birds from site B. The initial poor condition in house 1, due to the flooding, did not appear to affect adversely the incidence of salmonellae contamination during the period of rearing.

In the flocks under investigation the infection appeared to originate in the chicks because the environment was free from salmonellae at the time of placement while the fluff samples, clean eggs, and papers from the chick trays were contaminated. This is a similar situation to that experienced by Patterson and Gibbs (1977) and one of the flocks of Snoeyenbis *et al.* (1974). Samples were taken by these workers to establish the rate of contamination of the litter. In this investigation it was found that it was possible to isolate salmonellae from the litter within 24 h of placement. Such rapid colonisation of the litter was not found by Patterson and Gibbs (1977) who did not recover salmonellae until the third week and then they isolated the organism in all the following weeks. Similarly, Snoeyenbis *et al.* (1974) found a delay before salmonellae were isolated from the litter but there was no " die-out " reported by these investigators. At both sites A and B after the initial isolation the salmonellae were found in varying numbers.

There was a delay of 14 d before salmonellae were isolated from the water troughs at site B although at site A they were isolated within 7 d. The isolation of salmonellae from water troughs has been reported by others (Gordon and Tucker, 1965; Dougherty, 1976; Patterson and Gibbs, 1977), but none of these authors stressed the apparent importance of the findings. Water is potentially an important oral route of infection as 100 chicks will drink 4·5 l/d. In the houses monitored in this investigation salmonellae were isolated more frequently from the water than from the litter and so may be a major source of re-infection of the birds.

The food did not appear to be a source of contamination on these sites as only one load of food delivered to the houses was contaminated and this led to two food samples from the houses being contaminated. As the birds were 56 d old when the

TABLE 3

Salmonellae isolated from water troughs

Age of birds (d)	Site A				Site B			
	House 1		House 2		House 3		House 4	
	Areas[1]	Salmonellae isolated	Areas	Salmonellae isolated	Areas	Salmonellae isolated	Areas	Salmonellae isolated
7	1, 7	a, i	6	a	NF	...	NF	...
14	4, 7	s	5	m	6	a	1, 6	a
21	7, 8	a, i	1, 4	i	1, 2, 3, 6, 7, 8, 9	a	1, 2, 4, 5, 6, 8, 9	a
28	2, 4	a, i	1, 7	a, i	1, 2, 3, 5, 6, 7, 9	a	1, 2, 4, 6, 7, 8, 9	a
35	NF	...	2	i	6, 8	a	NF	...
42	NF	...	4, 7	s, [2]	NF	...	1, 2, 5, 6, 7, 9	a
49	8	i	1, 3, 7	a, [2]	4, 8	a	9	...
56	NF	...	...[1]	...	4, 7	a	...[2]	...
63	...[2]	...	...[1]	...	4, 5, 8, 9	a	...	...
70	...	...	...	...	2, 4, 6	a	...	...

a, *S. agona*; i, *S. infantis*; m, *S. montevideo*; s, *S. senftenberg*; NF, not found.
[1] See Fig 1.
[2] Birds slaughtered.

Dust

At site A dust was collected from the brooder hoods once sufficient quantity had accumulated. Table 4 shows that except for the isolation of *S. infantis* and *S. senftenberg* at day 35, only at the end of the rearing period (day 56) were salmonellae frequently recovered. A higher number of samples from the fans were contaminated at site B. *S. infantis* was isolated from the dust at site B, the only occasion on which this serotype was isolated at this site. *S. montevideo* was not found in the dust.

TABLE 4

Salmonellae isolated from dust

Age of birds (d)	Site A (eight brooder hoods)				Site B (fourteen fans)			
	House 1		House 2		House 3		House 4	
	Areas[1]	Salmonellae isolated	Areas	Salmonellae isolated	Areas	Salmonellae isolated	Areas	Salmonellae isolated
28	NT	...	NT	...	NF	...	1, 2, 3, 4, 5, 6, 8, 10, 13, 14	a, i
35	NF	...	1, 2	i, s	NF	...	NF	...
42	NT	...	NT	...	NT	...	NT	...
49	NF	...	NF	...	NT	...	NT	...
53	NT	...	NT	...	NF	...	1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 12	a
56	2, 7, 8	a, i, s	1, 2, 5, 6, 7, 8	a, i	1, 2, 3, 4, 8, 10, 12, 13, 14	a, i	...[2]	...

a, *S. agona*; i, *S. infantis*; s, *S. senftenberg*; NT, not tested; NF, not found.
[1] See Fig. 1.
[2] Birds slaughtered.

Foodstuffs

S. agona was recovered from the foodstuffs at the end of the rearing period at site A when 1 to 2 organisms/100 g were found in the hopper of house 1 and troughs in area 8 of house 2. On this occasion the same bulk food was used in the hoppers of houses 1 and 2. No isolations were made from the foodstuffs at site B.

Birds

Salmonellosis was not a problem during the rearing of the birds. However, Table 5 shows that there was a low incidence of infection of the birds at the point of slaughter. *S. agona* and *S. infantis* were isolated from both external sites and the organs of the birds.

Litter

Once the houses of site A were occupied, salmonellae were recovered from the litter within 24 h (see Table 2) of the chicks being placed. During the rearing period sporadic isolations were made from the litter of both sites A and B (Table 2).

Although house 1 was flooded before use, the incidence of salmonellae was no greater than in house 2 and after 21 d salmonellae were not isolated from house 1. In house 2 salmonellae were not detected after 21 d and re-appeared on the 49th day. *S. infantis*, *S. agona* and *S. senftenberg*, but not *S. montevideo* were recovered from litter on site A. At site B only *S. agona* was isolated, the contamination not being detected until 7 d. Although the initial incidence of salmonellae at site B was less than at site A, salmonellae were more frequently isolated from the litter of houses 3 and 4 than from that of houses 1 and 2. The higher number of isolates at site B could have been due to the improved method of isolation or a real increase in contamination. The spatial relationship of the serotypes and locations did not form a pattern but it is noteworthy that the incidence of contamination at the doorway was no higher than in the rest of the house.

TABLE 2

Salmonellae isolated from litter

	Site A				Site B			
	House 1		House 2		House 3		House 4	
Age of birds (d)	Areas[1]	Salmonellae isolated	Areas	Salmonellae isolated	Areas	Salmonellae isolated	Areas	Salmonellae isolated
1	6, 7	s, i	1, 7	s	NT	...	NF	...
7	7	a	3, 6, 7	s, a, i	5	a	2, 4	a
14	NF	...	2	s	1, 3	a	NF	...
21	3, 5, 6	a	3	s	3	a	6	a
28	NF	...	NF	...	6	a	4, 7	a
35	NF	...	NF	...	6, 9	a	1, 5, 7	a
42	NF	...	NF	...	9	a	1	a
49	NF	...	2, 6	a, i	1, 2, 8	a	1, 5, 8	a
56	NF	...	...[2]	...	NF	...	...[2]	...
63	NF	...	...	...	NF	...	...	...

a, *S. agona*; i, *S. infantis*; s, *S. senftenberg*; NT, not tested; NF, not found.

[1] See Fig. 1.

[2] Birds slaughtered.

Water troughs

Salmonellae were isolated from the water troughs after 7 d at site A and 14 d at site B. As can be seen from Tables 2 and 3 salmonellae were isolated from the water trough more frequently than from the litter. *S. agona* was the only serotype isolated at site B. At site A *S. infantis*, *S. montevideo*, *S. senftenberg* were isolated as well as *S. agona*. When the serotypes isolated from the water troughs and the litter are compared it can be seen that at site A the same serotypes were not recovered on the same dates from both situations. At site B only *S. agona* was isolated from the litter and water troughs. There was no relationship between the areas of the house where salmonellae were isolated in the litter and the water troughs.

Birds

Twenty-four hours before slaughter 10 visually healthy birds were taken from each house. They were killed by dislocating the neck and taken to the laboratory in individual polythene bags, the feet protected by separate polythene bags. The birds were examined by first removing the feet and adding one to each enrichment broth. A sample of the feathers was taken and then the bird dissected aseptically; the gizzard, liver, heart and intestine removed and each treated as a separate sample.

Laboratory examination

The samples or sub-samples from site A were divided into two portions and a volume equivalent to ten times the weight of the sample of selenite F broth added to the one portion and tetrathionate broth (Rolfe, 1946) added to the other. Both enrichment broths were incubated at 43 °C. At 24 h and 48 h a loopful of each enrichment broth was streaked onto brilliant green agar (Oxoid) and desoxychlorate agar (Oxoid) and after incubation at 37 °C for 24 h and 48 h respectively, six suspect colonies were picked off each plate and examined by conventional methods. Representative isolates were submitted to the Scottish Salmonella Reference Laboratory.

All samples from site B were pre-enriched in 250 ml nutrient broth (Oxoid) for all samples except the litter samples when 500 ml was added. After incubation at 37 °C for 24 h an equal quantity of double strength selenite F broth or double strength tetrathionate broth was added and the isolation continued as described for site A samples.

RESULTS

Salmonellae were not isolated from the stream and ditch when they flooded house 1 in February but after 21st March *Salmonella infantis* and *S. agona* were isolated from every water sample.

No salmonellae were recovered from the environmental samples, water troughs, litter and food in the houses on both sites before occupation. However, as shown in Table 1 salmonellae were isolated from fluff, unhatched eggs and papers from the trays in which the chicks were transferred to the house from the hatchery. The 1-d-old chicks at site A had been exposed to *S. agona*, *S. infantis*, *S. montevideo* and *S. senftenberg* while at site B only *S. agona* was identified.

TABLE 1

Number and types of salmonellae isolated from incubator debris and chick boxes

	Site A		Site B	
Material	House 1	House 2	House 3	House 4
Fluff	0/4	2/4 (a, i, s)	1/5 (a)	1/5 (a)
Chicks dead in shell (250 eggs)	50 (s, i)	50 (s)	...	...
Clear eggs (250 eggs)	40 (a, m, s)	10 (s)	...	...
Chick box papers (250 papers)	80 (a, s)	100 (a, i, m, s)	10 (a)	30 (a)

a, *Salmonella agona*; i, *S. infantis*; m, *S. montevideo*; s, *S. senftenberg*.

Litter

At site A twenty handfuls of the surface litter were taken from each area and at site B thirty handfuls per area. Polythene gloves were used to exclude contamination of the sample. The samples were transported to the laboratory in a polythene bag, where, after mixing, a sub-sample of 500 g was examined.

Foodstuffs

Samples of about 1 kg were taken from the bulk hoppers from both sites. At site A a handful of the material from each trough in each area was bulked in a polythene bag. No attempt was made to separate extraneous matter from the food stuff, the contents of the trough was considered as " food ". At the laboratory, after mixing, four sub-samples of 25 g were weighed out, hence the numbers of salmonellae present could be estimated (McCoy and Spain, 1969). The individual food troughs were not sampled at site B.

Water troughs

Two water troughs per area were sampled at site A and four troughs per area at site B. The troughs were swabbed with a sterile cellulose sponge (50 mm × 70 mm) using sterile polythene gloves to prevent contamination of the water during sampling. The sponges were drawn down the base of the trough and pressed into the seams and placed in pairs in 500-ml screw-capped jars for transport to the laboratory.

Dust

Samples of dust were taken from the brooders in each area of site A and from every fan on site B.

Chicks

Contamination of the chicks was detected by examining the papers from the plastic trays in which the chicks were carried to the house from the hatchery. Fluff samples and unhatched eggs were examined from the incubator in which the chicks were hatched.

Water

At site A, a 1-l sample of water was taken from the stream and the ditch on the same day as the houses were sampled. This was divided into ten 100-ml aliquots and equal quantities of double strength solutions of the enrichment broths added.

Environmental samples

After cleaning and fumigation 1 m^2 of the wall between each fan was swabbed using one cellulose sponge moistened with $\frac{1}{4}$-strength Ringers solution containing 1 g Teepol/l.

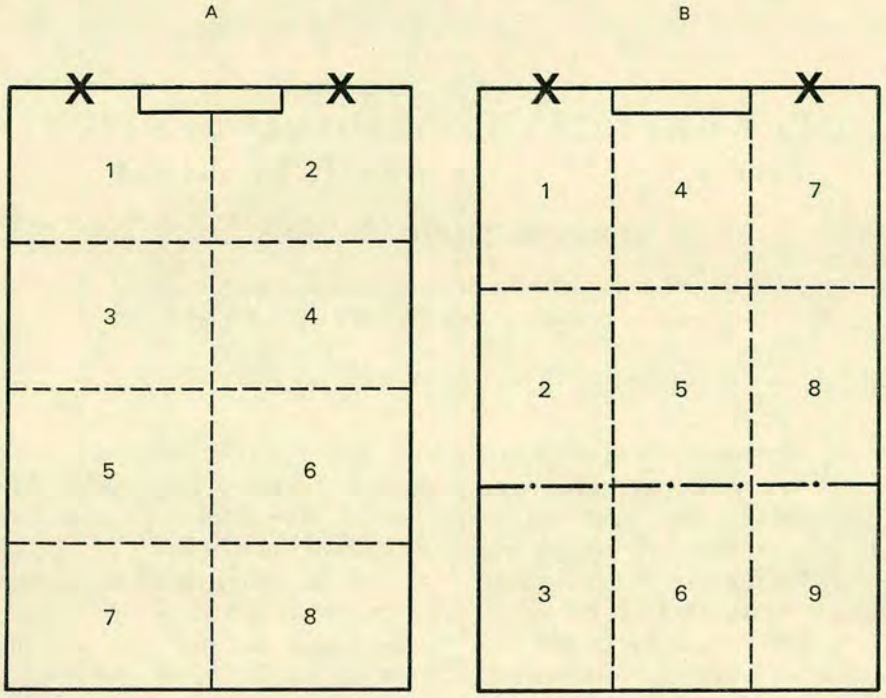

FIG.—Plans of sampling areas at: A site A, and B site B. X marks door positions and —·—·— indicates position of fence.

investigation. Site B was located on a north facing slope, the houses facing east. The houses were sited on two levels, the houses on the upper level being selected for monitoring, designated houses 3 and 4. All the houses were of the same design, the ventilation being by fourteen 600-mm fans and heating by seven centrally placed individual brooders, a background house temperature of 23 to 25·5 °C being maintained throughout the rearing period. All the houses had sawdust bedding, and galvanised iron water troughs. The feedstuffs were milled and pelleted on an adjoining farm.

The routine cleaning procedure was to remove from the houses all the movable equipment for hand cleaning and the litter was then removed. After a thorough cleaning with a pressure hose the concrete floors were sprayed twice with formaldehyde solution (10 g/l) and new litter placed in the houses and the movable equipment replaced. The houses were then fumigated by heating paraformaldehyde.

House 1 was flooded by the stream overflowing, 18 h prior to the planned time of placement of the chicks and the litter was replaced and the chicks put into the house 24 h after the scheduled date.

Sampling procedures

Sampling was carried out at 7-d intervals. On site A houses 1 and 2 were divided into eight areas for sampling (see Fig. A). At Site B there was a fence across each house and houses 3 and 4 were divided into nine areas (see Fig. B).

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THE OCCURRENCE OF SALMONELLAE DURING THE REARING OF BROILER BIRDS

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1. The incidence of salmonellae was studied in two broiler houses at each of two commercial sites in an integrated commercial enterprise.

2. Salmonellae were not isolated from the empty, cleaned and fumigated houses and only on one occasion from the foodstuffs.

3. Salmonellae were isolated from the environment of the chicks and spasmodically from the litter, water troughs and dust.

4. The incidence of infection of the chicks did not influence the number of isolations of salmonellae from the environment of the birds during rearing.

5. Water in the water troughs rather than foodstuffs appeared to be the major oral route of infection or re-infection of birds during rearing.

INTRODUCTION

In the last decade there has been an increase in the number of cases of salmonella food poisoning where poultry has been proved to be the source, or has been implicated by circumstantial evidence (Lee, 1973). Whether the increase has been due to a greater awareness of this source, or an increase in communal eating and an increase in poultry consumption, or improved methods for isolating salmonellae remains a matter for conjecture. Although the degree of contamination of carcasses may be reduced during processing (Morris and Wells, 1970; Morgan-Jones, 1977), it is desirable that birds entering the packing station are not infected. In order to reduce the levels of infection in broiler flocks the foci of contamination need to be identified so that control measures can be considered. This investigation was undertaken to determine the relative importance of the sources of salmonellae in a commercial integrated broiler enterprise in Scotland.

MATERIALS AND METHODS

Broiler houses

The two houses involved were on two different sites. Site A had five houses facing west on a flat area with a stream on the north side and a ditch on the west side. The second and fourth houses, designated houses 1 and 2, were selected for

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TABLE 5

Incidence and serotypes of salmonellae at pole-barns for batch 2

Weeks	Litter	Water	Dust	Foodstuff
11	1/10 (w)	0/110	0/10	0/1
13	1/10 (w)	1/10 (w)	0/10	0/1
15	1/10	0/10	0/10	0/1
17	0/10	0/10	0/10	0/1

w: *S. worthington.*

DISCUSSION

Vigilance on the part of the management in monitoring the bacteriology of the hatchery fluff has shown that in this enterprise the breeding stock were free from salmonella contamination. So, as with the study of Zechc *et al.* (1977) the contamination was not brought in with the poults. The spread of contamination from one batch of animals to the next due to faults in cleaning has been shown for calves (Anon, 1965; Morgan-Jones and J. I. Kelly, personal communication) and turkeys (Kumar *et al.*, 1971). In this investigation the error in cleaning routine was traced to the water troughs being filled prior to fumigation. In the case of Kumar *et al.* (1971) the carry-over was due to salmonellaé in the dust but in both instances the contamination persisted throughout the rearing of the turkeys.

The sporadic isolation of salmonellae from the litter and dust samples was a similar picture to that found in a monitoring exercise reported by Morgan-Jones (1980) for chicken houses. The effect of greater attention to detail in the hygiene programme is seen in the relative freedom from salmonellae in the birds in batch 2.

The food did not appear to be the source of contamination because the samples from the mill were negative but the possibility of contamination being re-cycled in the houses by the food being contaminated during its passage through the house is a disadvantage with automatic feeders. A similar observation was made by Patterson and Gibbs (1977) in chicken houses. Gordon and Tucker (1965), Dougherty (1976) and Morgan-Jones (1980) have isolated salmonellae from water troughs. The results of this investigation confirmed the importance of water as a source of contamination and subsequent re-contamination of the birds.

The conditions of rearing of the stags at the pole-barns are very different from those in the environmentally-controlled houses. Batch 1 was reared in September to November and batch 2 in January to February. Therefore the lower ambient temperature may explain the lower incidence in salmonellae in the second batch but the increased attention to hygiene by the staff possibly was a major factor. The new troughs used for batch 2 were more easily maintained in a visibly clean condition which probably resulted in just one isolation of *S. agona* from the water as compared with the frequent isolation in batch 1.

This investigation showed that the incidence and sites of contamination of salmonellae during the rearing of turkeys are very similar to those found in the rearing of chickens. In addition, it has shown that contamination can be carried from one batch to subsequent batches by faulty hygiene failing to render the house free from this organism.

TABLE 3

Incidence and serotypes of salmonellae in batch 2

Age of poults (d)	Room A			Room B			
	Litter (500 g)	Water troughs	Dust	Litter	Water troughs	Dust	Foodstuff
1	...	NT	NT	...	NT	NT	NT
7	0/1	0/11	0/1	0/1	0/12	0/1	0/1
21	0/1	0/16	0/1	0/1	0/16	0/1	0/1
45	0/1	0/12	0/1	1/1 (a)	0/11	0/1	0/1
60	0/1	0/12	0/1	0/1	0/11	0/1	0/1
74	0/1	0/12	0/1	0/1	0/11	0/1	0/1

NT: not tested, a: *S. agona.*

shows that with the exception of one isolation of *S. agona* from the litter at 45 d the house was free from salmonellae.

In the pole-barns there were greater opportunities for the birds to be contaminated from external sources although starlings and mice recovered from the site were always negative for salmonellae. Samples from the cleaned pens were also always negative. The water troughs used for batch 1 were only hosed down before being placed in the fresh pen. The design of the building precluded removal of the dust after each batch, the houses only being brushed annually. As can be seen from Table 4

TABLE 4

Incidence and serotypes of salmonellae at pole-barns for batch 1

Age of stags (weeks)	Litter	Water troughs	Dust	Foodstuff
11	0/4	2/4 (i, w)	1/4 (a, w)	0/1
13	1/4 (w)	0/4	0/4	0/1
15	0/4	0/4	1/4 (w)	0/1
17	3/4 (w)	1/4 (w)	1/4 (w)	0/1
19	3/4 (w)	1/4 (w)	0/4	0/1
21	3/4 (w)	1/4 (w)	0/4	0/1
22	3/4 (w)	1/4 (w)	0/4	0/1
24	0/4	3/4 (w)	1/4	0/1

a: *S. agona*, i: *S. infantis*, w: *S. worthington.*

the birds were subjected to a high level of contamination yet salmonellae were not isolated in the viscera and neck skins of 25 carcases examined at slaughter.

As at the rearing unit the results of batch 1 were discussed with the Site Manager of the pole-barns prior to receipt of batch 2. The management replaced the wooden water troughs with galvanised troughs after batch 1 and more attention was paid to maintaining them visibly clean. As can be seen in Table 5 the level of contamination with salmonellae in batch 2 was considerably lower than in batch 1 and no salmonellae were detected in 25 carcases examined at slaughter.

Laboratory examination

All samples were examined in the manner described by Morgan-Jones (1980) for site B.

RESULTS

Routine examination of fluff samples from all batches of poults incubated at the hatchery had always proved negative for salmonellae confirming the results shown in Table 1 that the poults at placement were free from salmonellosis. Table 1 also shows

TABLE 1

Incidence and serotypes of salmonellae at time of placement of poults

	Batch 1		Batch 2	
Materials	Room A	Room B	Room A	Room B
Swabs of building	0/10	0/10	0/10	0/10
Litter	0/1	0/1	0/1	0/1
Water troughs	2/3 (w)	1/3 (w)	0/3	0/3
Poult-box papers	0/50	0/50	0/50	0/50

w: *S. worthington.*

that initially the structure of the rearing houses and the litter were negative but the water from the troughs was contaminated. When these results were discussed with the Site Manager it was discovered that water had been put into the troughs before fumigation. This coupled with ineffective cleaning of the troughs had led to a carry-over of salmonellae to that batch. As can be seen in Table 2, within 24 h salmonellae were detected in the litter and then sporadically throughout the time the birds were in the house. Samples of foodstuff from the mill were always negative and the salmonellae isolated from the food in the house were possibly due to contamination from the feeder mechanism resulting in recycling of infection in the houses.

When the house was being prepared for batch 2 the troughs were dry when fumigated and the water added as the poults were introduced. As can be seen in Table 1 no salmonellae were isolated when batch 2 was placed in the house and Table 3

TABLE 2

Incidence and serotypes of salmonellae in batch 1

Age of poults (d)	Room A			Room B			
	Litter	Water trough	Dust	Litter	Water trough	Dust	Foodstuff
1	5/8 (a, w)	NT	NT	7/8 (a, w)	NT	NT	NT
15	1/1 (s, w)	2/5 (a, s, w)	NT	1/1 (s, w)	3/5 (a, w)	NT	1/1 (w)
30	1/1 (w)	1/5 (a)	1/1(s)	0/1	0/1	1/1(w)	1/1(w)
40	1/1 (w)	5/5 (w)	0/1	1/1 (s)	1/5 (w)	1/.1 (s)	0/1
64	0/1	1/5 (a, w)	1/1 (a, w)	1/1 (w)	4/5 (w)	1/1 (a, w)	1/1 (a)
78	1/1 (w)	1/5 (a)	1/1	0/1	3/5 (w)	0/1	0/1

NT: not tested, a: *S. agona*, s: *S. senftenberg*, w: *S. worthington.*

MATERIALS AND METHODS

Rearing houses

The turkeys were reared in an environmentally-controlled house until 10 to 12 weeks when the females were removed for slaughter and the males transferred to pole-barns (straw yards) for up to a further 14 weeks.

The concrete-floored rearing houses were divided into two rooms by a wooden partition in which there was a door. These are designated as rooms A and B. The ventilation of each room was by seven fans and heating by two rows of four individual brooders maintaining a background temperature of 60 to 70 °C during the rearing period. Initially the litter was of sawdust but after 4 weeks this was overlaid with chopped home-grown barely straw. Water was supplied from a borehole into plastic conical drinkers. The foodstuffs were milled and pelleted on the farm and fed to the birds by an automatic feeder (Big Dutchman).

The earth-floored pole-barns were naturally ventilated, protection from the elements being provided by polythene sheeting in the upper section of the walls. Unchopped barely straw was used for litter and water was provided in wooden troughs for the first batch, but these were replaced by galvanised metal troughs for the second batch. Mixed meal was offered *ad libitum* in fixed wooden troughs along one side of the pens.

The management of the pole-barns was separate from the rearing farm. The batches of birds from different rearing houses were sometimes mixed in the pens of the pole-barns and the length of time spent here depended on the marketing demands.

Cleaning routine

At the rearing site the movable equipment was removed from the house for hand cleaning and the litter was removed mechanically. After thorough cleaning with a high-pressure hose, the walls and floor were sprayed with a phenolic disinfectant and the house rested for at least 2 weeks. New litter was then placed on the floors and the movable equipment replaced. Two days before the poults were introduced the houses were fumigated by the paraformaldehyde method.

The pens in the pole-barns were cleaned by moving the water troughs and then the litter. After thorough brushing, the floors were covered with slaked lime for at least 2 weeks. The pens were then relittered and the water troughs replaced.

Sampling procedures

Samples from the environment, water troughs, litter and poult-box papers were taken at the same time as the poults were introduced. The litter under the brooders was sampled at 24 h and further samples of the litter, foodstuffs, dust and water troughs were taken at 2-week intervals. No dead poults were tested, but ten starlings and six mice caught on the site were tested. Viscera and neckskins were sampled at the turkey packing station at the time of slaughter. The methods used were as described by Morgan-Jones (1980). Sampling was continued for two batches of birds.

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THE PRESENCE OF SALMONELLAE DURING THE REARING OF TURKEYS

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1. The incidence of salmonellae was investigated in two batches of turkeys in the environmentally-controlled houses and naturally ventilated pole-barns of a commercial turkey enterprise.

2. The poults were free from contamination on arrival at the rearing house but were contaminated via the water supply from troughs which had been ineffectively fumigated.

3. Foodstuffs did not appear to be a source of contamination.

4. The spread of salmonellae in this turkey rearing enterprise was very similar to that in chicken rearing houses.

INTRODUCTION

The practice of further processing turkey carcasses for sale as portions or burgers, sausages etc. has increased recently but still the greater proportion of turkey meat is used in the catering industry. As food consumed at social functions is more frequently implicated in food poisoning outbreaks than home-cooked foods the impact of salmonella contamination in turkeys has a disproportionately greater effect on public health than would be expected from the numbers reared. For instance in Scotland in the period 1975 to 1980 there were 76 outbreaks of salmonella food poisoning in which poultry were implicated and of these 24 were traced to turkeys (J. C. M. Sharp, personal communication). However, the ratio of turkeys reared to broiler chickens reared is approximately 1 : 10.

Although a great deal of work has been carried out on the ecology of salmonellae in chickens (Snoeyenbos *et al.*, 1974; Dougherty, 1976; Bains and MacKenzie, 1974; Patterson and Gibbs, 1977; Morgan-Jones, 1980) little similar work has been undertaken at turkey farms. The rearing of turkeys differs from that of chickens in that the former require a lower environmental temperature. Turkeys are also grown for longer periods than broilers, often up to 24 weeks, but the increased popularity of the small ("mini") turkey has led to increasingly larger numbers of birds being killed at 8 to 10 weeks. These differences in husbandry may have altered the rearing environment of the birds such that similar conditions in chicken and turkey enterprises can no longer be assumed. The reported investigation was undertaken to examine the spread of salmonellae in turkeys reared under a commercial system.

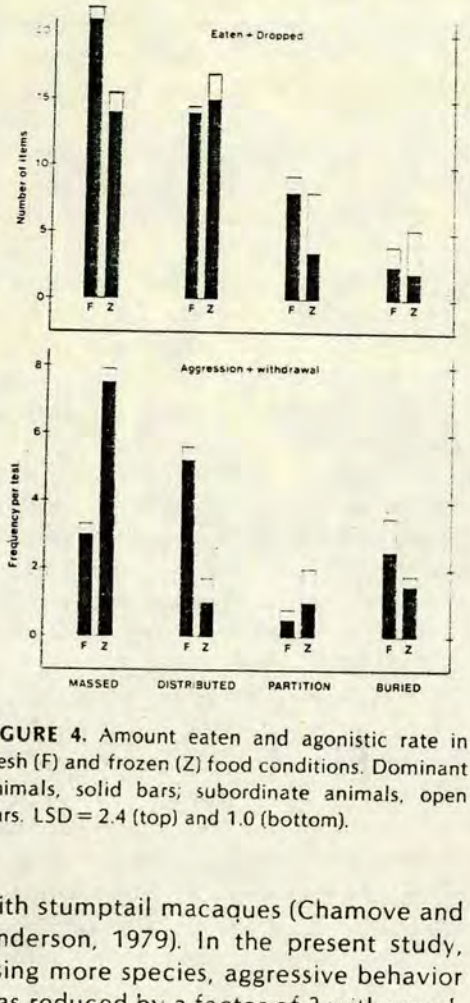

FIGURE 4. Amount eaten and agonistic rate in fresh (F) and frozen (Z) food conditions. Dominant animals, solid bars; subordinate animals, open bars. LSD = 2.4 (top) and 1.0 (bottom).

with stumptail macaques (Chamove and Anderson, 1979). In the present study, using more species, aggressive behavior was reduced by a factor of 3 with woodchips and by almost 10 times with grain or mealworms added to the litter. All negative behavior decreased by a factor of over 5 when food was added to the woodchips. Time spent on the ground almost doubled with woodchips, and more than doubled when food items were added to it. These effects occur in monkeys of various ages. Figure 5 illustrates a group of stumptail monkeys foraging through woodwool, another type of litter we are evaluating. We have observed that it does not "pack" in the same way as woodchips do, and may therefore be left down longer.

In addition to searching through the two types of litter, juvenilesalso engage in playful gymnastics in them, more so than on a bare floor, and more on wood wool than on woodchips.

In addition, there is no evidence that using woodchips presents a health hazard. As the litter matures, the wood chips become increasingly more inhibitory to bacterial survival. This self-sterilizing action makes it likely that the mere presence of an absorbent litter greatly reduces the probability of disease spread due to fecal contamination.

The freezing of food also has advantages in certain situations, leading to improved distribution and less fighting. This is particularly true when the dominant animals cannot "control" the food sites. Distribution of the food *per se* in a small enclosure may not reduce aggression, because the dominant animals may try to monopolize most of the food that they can see. One method of reducing the dominant animals' ability to control the food—burying it—resulted in improved distribution and prolonged feeding times. We regularly bury small food and non-food items in the woodchips, which the monkeys seem to enjoy discovering.

In conclusion, we recommend deep litter as one technique of enhancing conditions for captive primates. It has real potential for promoting good health and induces positive kinds of behavior among species that invest a great deal of time and energy in foraging in their natural environment.

Acknowledgments

The authors wish to thank M. Stevenson for help and permission at Edinburgh Zoo, and Miss A. Coles for technical assistance with the microbiological analysis.

References

Anonymous (1978) Microbiology associated with hatching, rearing and process-

massed in two piles or distributed evenly over the floor area. (3) To assess the effects of inter-animal visibility, the food was either distributed in the outside area where all subjects could see one another when feeding, or distributed over the same area inside where four opaque dividers with openings restricted visual contact among subjects. (4) To assess the effects of visibility of food, the food was either distributed on a bare area of the outside floor as above or buried under woodchips in the same area.

In all conditions two tests were run, one using fresh food, the other using frozen food. In all tests except experiment 1 the food used was apple. In each test the total weight of the food, cut into 45 pieces, was 1.25 kg.

Four measures were recorded on nine selected animals. The measures were (1) the number of food items eaten, *i.e.*, picked up and more than one bite taken from it; (2) the number of items eaten plus sampled, *i.e.*, dropped after only one bite was taken from it; (3) the number of agonistic interactions; and (4) the time that elapsed until all of the food had been consumed.

The analysis used analyses of variance with subjects divided into dominant (N = 2) and subordinate (N = 7) subgroups. All results reported below are significant beyond the .05 level unless specifically stated otherwise.

Results

Figure 3 illustrates the major significant differences observed. Under the condition in which food was distributed, freezing the food reduced aggression by a factor of 3 but had only a slight positive effect on distribution of food among the animals. In general, as the possibility of the dominant monkeys seeing and controlling all the food items decreased (under the conditions displayed from left to right in Fig. 4), the amount consumed by the dominants decreased, the amount eaten by the subordinates increased, and aggression was reduced. This effect was accentuated when the food was frozen.

The behavior of the dominant pair was more complicated. When the food was massed in two piles and frozen, the long feeding time led to aggression as the dominants attempted to control the two piles. When the food was distributed, fresh, and visible, aggression was also common due to attempts at control by the dominant subjects. Freezing the food reduced this aggression.

The test conducted inside, where dividing partitions restricted inter-animal visibility, was over in 2 minutes when fresh food was used, and aggression was infrequent. Aggression was slightly increased in the test using frozen food, which lasted much longer—24.3 minutes. Corresponding durations from the tests done outside were 6.4 and 19.0 minutes. To provide some perspective on these values, an adult stumptail eats an apple in about 1.8 minutes and a banana in about 0.9 minutes. A frozen apple or banana takes about six times as long to eat.

In the tests involving three types of distributed food, the dominants ate relatively more of the two preferred foods when it was offered fresh than when it was frozen, but not of the carrot. Aggression by the dominant monkeys was over four times greater for banana and apple when these were fresh than when they were frozen, but aggression was roughly equal (when fresh) and much lower (frozen) for the carrot.

Discussion

The results of the present studies clearly show that there are advantages to using woodchips as a substrate for monkeys. These data thus support the conclusions reached in a previous study

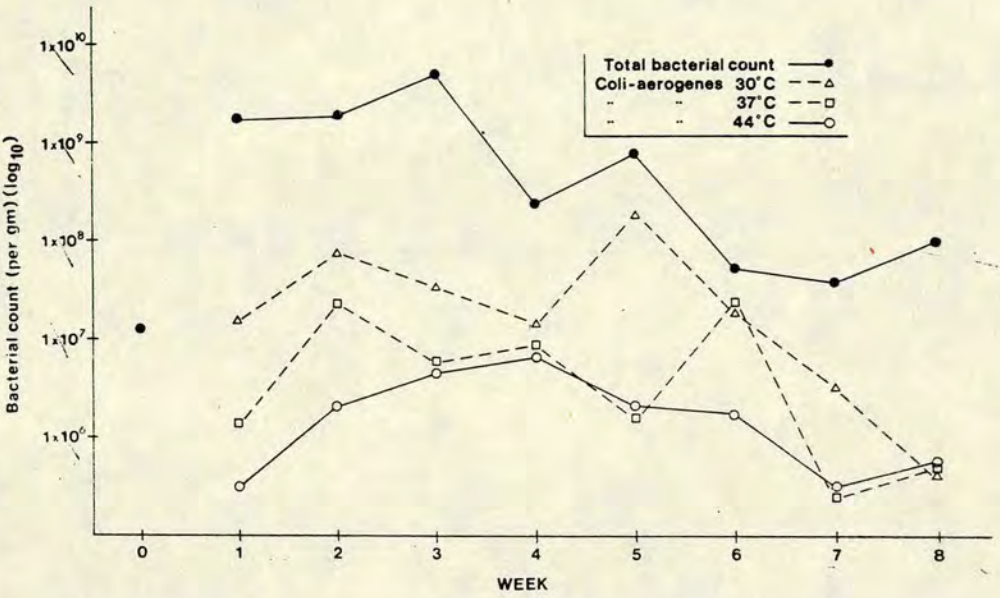

FIGURE 3. Microbiological analysis of litter.

rose from 2.9 x 10^4 per g in week 0 to a maximum of 2.4 x 10^6 in week 1, then gradually declined to a minimum of 4.3 x 10^1 by week 8 (weeks 2 to 7: 2.4 x 10^4, 3.3 x 10^4, 3.3 x 10^4, 4.6 x 10^3, 1.5 x 10^4, 1.1 x 10^3, 2.3 x 10^2). It is of interest here that the monkey litter was as inhibiting to salmonellas as is poultry litter (Morgan-Jones unpublished data).

These results show that the use of litter will not increase the risk of bacterial disease transmission and in fact appreciably reduces that risk. We have observed that after a period of about 12 weeks the monkeys spend less time on the litter and are less interested in searching through it. This behavioral criterion is useful in the scheduling of litter changes; we have decided that renewal every 4 to 6 weeks is optimal at our population densities.

Study 3

Fresh fruit and vegetables are usually given to captive monkeys to relieve the boredom of standardized diets. Two problems that often occur when feeding group-housed animals are: (1) the dominant animals are able to expropriate a disproportionate amount of the food, and (2) the food is eaten too quickly. We have observed that feeding solidly frozen fruits and vegetables to monkeys leads to better distribution and longer feeding times (Chamove, 1981), and have been using this method for the past 7 years with no ill effects. Study 3 was carried out to quantify and verify our earlier observations.

Methods

The Stirling colony group of 25 stumptail macaques was used. Their ages ranged from 6 months to 8 years, with a mode of about 2 years. Four experimental comparisons were made. (1) To assess the influence of incentive, three foods were offered in decreasing order of preference—banana, apple, and carrot. (2) To assess the effect of manner of distribution, food was either

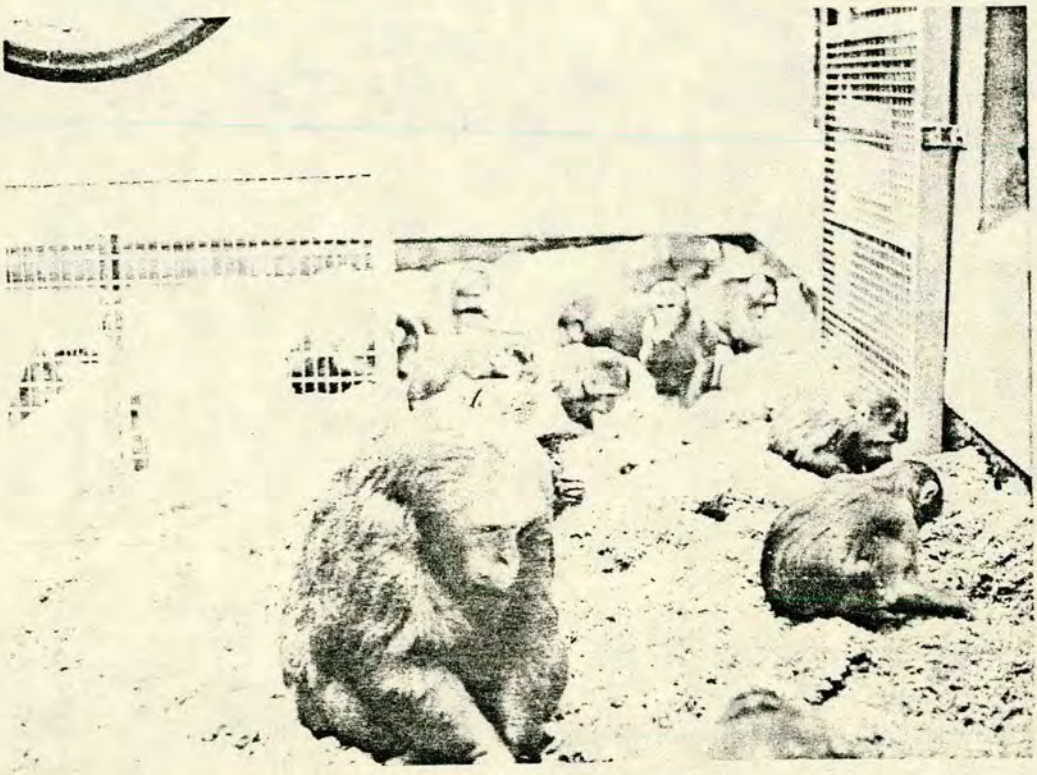

FIGURE 2. Macaques search through litter for grain in the test area.

and then shaking and incubating it at 22°C for 48 hours. The numbers of salmonella organisms in the litter after storage were estimated using the method described by Morgan-Jones (1982).

Results

Correlations of times (age of litter, expressed in weeks) with bacterial counts ranged from −.41 for the total count to −.60 for salmonella, and between −.70 and −.76 for the three coliforms. Although pH and percentage of dry matter correlated highly with week number ($r = +.65$ and $-.59$, respectively) and also with one another ($r = -.60$), the correlation between pH and week number did not seem to be caused by moisture content, since partialling out percentage dry matter did not substantially reduce the correlation ($r = +.50$).

Similarly, with one exception the correlation of bacterial inhibition with week number was not accounted for by either moisture content or pH of the litter. Partialling out the variance due to percentage of dry matter reduced the bacterial correlation with week number by only .04, on average; partialling out pH reduced it by only .03, except for the 37°C test (.14) and the total count, where it actually increased by .25.

It is clear from Fig. 3 that the total bacteria count decreased over the weeks. This was also true for coliforms isolated at 30°C, which include coli-aerogenes of both animal and nonanimal origin; 37°C, which reflect coliform bacteria of fecal origin; and 44°C, which reflect coliforms of very recent fecal origin. The survival tests for inoculated salmonella showed a similar pattern of reduced survival over the weeks. The numbers of salmonella

Method

Twenty-five stumptail macaques (*Macaca arctoides*), with a mean weight of 6.5 kg, were housed in an area composed of an indoor colony room and two outside areas of 33 sq m and 20 sq m respectively (described and illustrated in Chamove, 1981). All cages were interconnecting, and the animals were free to roam throughout the three areas. The outside pens were covered with mesh and partly covered with clear plastic. The floor area of each of the outside pens was covered with three 40-kg bales of woodchips. Twelve samples were taken from weeks 0 to 8 during July and August 1981. The samples were collected randomly from five different areas of a pen and mixed. Figure 2 illustrates members of a group of 25 stumptail macaques foraging through woodchips in an outside pen. Chips are covering only half of the pen floor.

Microbiological Analysis. One gram of the litter was taken, and serial dilutions were prepared using 1/4-strength Ringer solution (Oxoid no. BR 52) as the diluent. Appropriate dilutions were plated on nutrient agar (Oxoid no. CH 3) using standard techniques (Ministry of Agriculture, Fisheries and Food, 1968) Coli-aerogenes bacteria were counted at 30°C (Meynell and Meynell, 1970), using MacCartney broth (Oxoid no. CH 5a). All tubes showing acid and gas production after 48 hours were subcultured into duplicate tubes of fresh media; one tube was incubated at 37 ± 1°C, and the other at 44 ± 0.25°C.

Because salmonella is such a common and serious disease-producing organism in monkeys (Chamove *et al.*, 1979), the inhibiting effect of the litter on *Salmonella typhimurium* was assessed by inoculating approximately 10^3 organisms into 1 g of litter in a MacCartney bottle

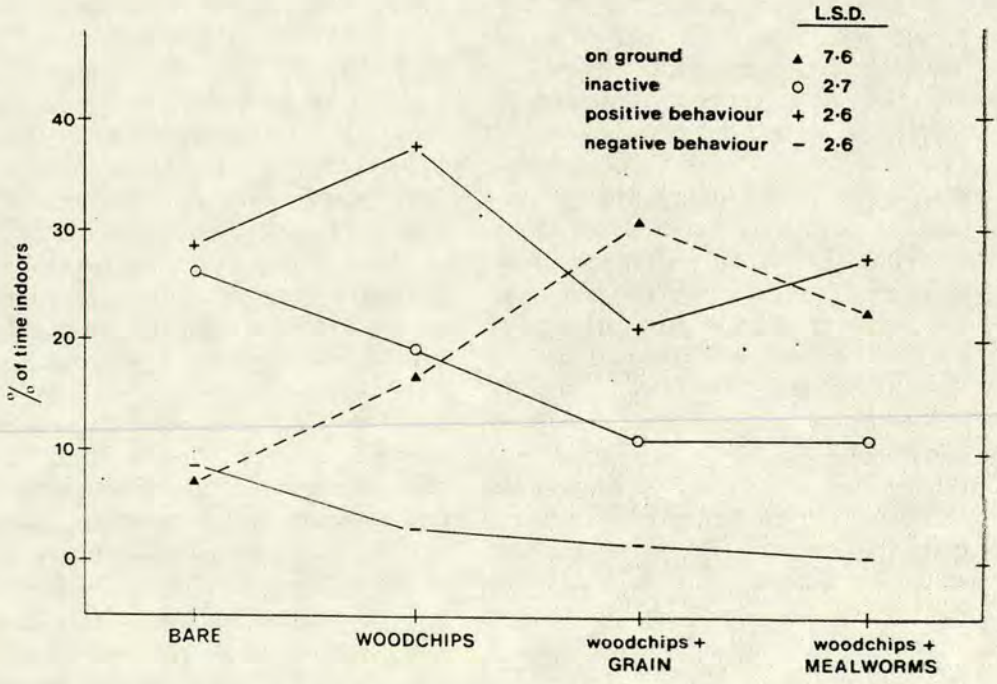

FIGURE 1. Behaviors as percentages of time when subjects were visible. Positive = affiliation + play, negative = agonistic + abnormal. The Fisher's LSD values are for the condition X behavior interaction.

tions, but this was not true of the two positive behaviors.

With woodchips, the relative proportion of affiliative behavior making up the positive category decreased as the environment provided was made more interesting; play was 3 times more frequent than affiliation in the bare condition, 5 times more frequent in the woodchips-only condition, and 8 times more frequent in the woodchips + food conditions. With woodchips, the subjects showed less negative and more positive behavior, in comparison with the bare condition. Grain added to the litter reduced the level of positive behavior, probably because of its distracting effects. The activity analysis showed significant effects of species X condition, and condition X behavior (both $P < .001$).

Because sleep rarely occurred, only percentage of time spent inactive is plotted in Fig. 1. The provision of woodchips decreased inactivity.

These results suggest that the mere presence of litter leads to positive behavioral changes, even after the novelty effects of its presence have passed. All species were less inactive; all except squirrel and vervet monkeys showed more play; all except capuchins engaged in a lower frequency of abnormal and agonistic behaviors; and all except marmosets spent more time on the ground foraging. The addition of grain or mealworms to the woodchips greatly increased the time spent on the ground, reduced inactivity, reduced play and affiliative behaviors, and tended to reduce aggression even further than with litter alone. Grain was particularly attractive to the stumptail macaques, lemurs, and vervet monkeys, while mealworms were particularly attractive to the tamarins and moustached guenons. This effect is shown in Table 1, which gives the condition that produced the greatest amount of time on the ground for each species.

Study 2

Study 1 confirmed and extended the finding that the use of woodchip litter with captive monkeys leads to positive behavioral changes. Furthermore, in our previous report the chips were shown to be inexpensive; after 6 weeks, odor was less than with bare floors, and the animals and walls appeared cleaner when woodchips were provided than when there was no floor covering but daily cleaning was performed (Chamove and Anderson, 1979).

One criticism of using litter with monkeys focuses on the danger of a buildup of disease, with the implicit assumption (Department of Health, Education, and Welfare, 1972) that the longer the litter is left down, the greater the danger. However, evidence from research on poultry litter suggests precisely the opposite, by demonstrating that mature litter is inhibitory to many disease organisms as well as to yeasts and molds (Fanelli, 1970; Snoyenbos, 1967; Tucker, 1967; reviewed in: Anon. 1978; Botts *et al.*, 1952; Duff *et al.*, 1973; Olesiuk *et al.*, 1971).

Chicks reared on old litter have lower mortality and grow more rapidly than controls. In addition, their eggs show increased hatchability (Botts *et al.*, 1952). The mere presence of old or new litter was shown by Duff *et al.* (1973) to eliminate the spread of salmonella among experimentally infected chicks. Although salmonellas survive for 3 to 4 weeks in feces (Berkowitz *et al.*, 1974), in used litter they are substantially destroyed within 3 to 5 days (Olesiuk *et al.*, 1971). The mechanism of salmonellacidal action is unclear, but there are suggestions that the increased moisture content (up to 20 percent), coupled with the high ammonia concentration and resulting alkalinity, are the critical factors (Turnbull and Snoyenbos, 1973). Study 2 assessed the potential for the spread of disease in litter used with macaque monkeys.

in the woodchips to the mouth. All scores were converted to a percentage of the intervals during which the subject was visible, *i.e.*, indoors. The data were analyzed using analyses of covariance. The percentage of time each subject was observed on the ground on the first 2 control days, the *bare* condition, was used to obtain a measure of arboreality, which was then used as a covariate (see Table 1).

Three analyses of covariance were performed. All included species (N = 8) and condition (N = 4) as factors. In addition, percentage of time spent inactive or asleep was used as a repeated measure in one analysis, as were "negative" behaviors, *i.e.*, aggression, fear, and abnormal activities, while "positive" behaviors, *i.e.*, play and affiliation, were employed in the second analysis. The third analysis used percentage of time on the floor, percentage of time engaged in foraging, and time spent outside as repeated measures. Alpha was set at .05, and all reported differences are significant beyond this level unless specifically stated otherwise. The Least Significant Difference (LSD) method was used to further evaluate significant effects.

Results

The results from all three analyses suggested that the addition of woodchip litter altered behavior. Surprisingly, the covariate had little effect: its largest beta estimate was only 0.20 for the analysis of foraging, indicating that the effect of the woodchip litter was not related to the degree of arboreality of the species. The forage analysis (Fig. 1) revealed two interesting effects (condition X behavior, and species X condition X behavior, both $P < .001$): (1) All species spent more time on the ground when it was covered with woodchips than when it was bare, and (2) when grain was incorporated into the litter, a further increase was noted. Since the foraging scores were very similar to the scores for the time spent on the ground, only the latter are plotted.

The social behavior analysis showed a significant condition X behavior effect ($P < .005$), and a significant species X condition X behavior interaction ($P < .05$). The positive and negative behavior scores are plotted in Fig. 1. Plots of the observed frequency of the two negative behaviors were parallel for the four sets of condi-

TABLE 1. Time on the ground and agonistic behavior in eight species in different conditions

Species	N	Time on ground in bare condition (%)	Time on ground in most effective condition (%)	Time exhibiting agonistic behavior (%)	
				BARE	WOODCHIPS
Guenon	8	39	68*	.20	.09
Vervet	4	17	26*	.11	.02
Lemur	3	9	87	.14	.10
Stumptail	6	8	80	.63	.18
Squirrel	7	5	13*	.20	.01
Capuchin	7	1	28	.13	.14
Tamarin	4	2	14	.52	.10
Marmoset	3	0	11	.40	.06

*In these 3 cases, the most effective condition was woodchip + mealworm; otherwise, it was woodchips + grain.

ful for the animal, leading to abnormal behaviors (Dawkins, 1980; Hediger, 1968; Meyer-Holzapfel, 1968). In captivity, food is usually presented once or twice per day, and it is therefore located and consumed in a short time. This contrasts with the extensive amount of time, up to 70 percent, that is spent in foraging activities in the wild (see references in Clutton-Brock, 1977; Harding and Teleki, 1981).

A second argument for the use of litter is an aesthetic one. Waste products are normally avoided by monkeys, but this is difficult when wastes are excreted onto solid floors. If monkeys avoid spending time on the floor of their cage because it is soiled, the area is being used inefficiently. Alternatively, the monkeys may be forced to spend time on a floor which they find aversive. Litter can serve to cover and absorb urine rapidly, and decompose feces. This study is an attempt to generalize the results of our previous pilot study of woodchip litter using stumptail macaques (Chamove and Anderson, 1979) to a variety of other primate species.

Method

The seven species of monkey and one prosimian that were studied were moustached guenons (*Cercopithecus cephus*, N=8), vervets (*C. aethiops*, N=4), ring-tailed lemurs (*Lemur catta*, N=3), stumptail macaques (*Macaca arctoides*, N=6), squirrel monkeys (*Saimiri sciureus*, N=7), black-capped capuchins (*Cebus apella*, N=7), red-bellied tamarins (*Saguinus labiatus*, N=4), and common marmosets (*Callithrix jacchus*, N=3). All were housed in Edinburgh Zoological Gardens, with the exception of the tamarins who were housed in a room in the Stirling University Psychology Primate Unit. The seven Edinburgh groups lived in indoor-outdoor enclosures. The outdoor areas contained dead trees and either grass or gravel on the ground. The floors of the indoor areas were of epoxy cement, and only this area was used for the study. Only the stumptails and tamarins had previous experience with woodchips on the floor.

Four conditions were studied: (1) baseline, *i.e.*, bare floor; (2) woodchips on the floor; (3) woodchips plus grain; and (4) woodchips plus mealworms. Two days of observation were conducted under the first three conditions and 1 day under the fourth. Following the 2 days of baseline observation, new woodchips were spread on the floors to a depth of approximately 4 cm. One week later, observations were undertaken under this, the *woodchip* condition. On the following day, 500 g (approximately 800 cc) of mixed grain was scattered and raked into the woodchips, and 30 minutes later the group was tested (see below for the testing methodology). This procedure was repeated the following day, using one-third of this amount of grain. These 2 days constitute the *woodchip + grain* condition. The grain mixture contained primarily millet seeds, with a small amount of peanuts, sunflower seeds, dried currants, wheat, and kibbled corn. The following day, five mealworms per animal were scattered onto the litter, and 30 minutes later the group was observed in this *woodchip + mealworm* condition.

Each test involved one experimenter monitoring the group for 20 minutes between 2 and 4 p.m. A metronome sounded every 10 seconds, and any behavior occurring during each interval was noted once. Threats, rough grabbing, and biting were recorded as aggression; grimaces, cowering, and fleeing were scored as fear. Stereotyped movements, bizarre postures, and self-aggression constituted "abnormal" behaviors. Affiliative behavior involved grooming or huddling with another animal. Foraging was defined as manipulating the woodchips and intermittently transferring items found

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Deep Woodchip Litter: Hygiene, Feeding, and Behavioral Enhancement in Eight Primate Species

Arnold S. Chamove, James R. Anderson, Susan C. Morgan-Jones, and Susan P. Jones

Sixty-seven animals from eight primate species were used to assess improved husbandry techniques. The presence of woodchips as a direct-contact litter decreased inactivity and fighting, and increased time spent on the ground. Placing food in the deep litter led to further behavioral improvement. The use of frozen foods improved food distribution and reduced fighting in most situations, especially when it was buried in the litter. With time, the litter became increasingly inhibitory to bacteria. The results suggest that inexpensive ways of increasing environmental complexity are effective in improving housing for primates.

Introduction

A desirable objective in the management of captive animals is the creation of an environment adequate for the animals' physical and emotional needs. This is especially true for nonhuman primates in whom social, physiological, and intellectual pathologies result when important environmental considerations are neglected (McGrew, 1981). Environmental enrichment can be achieved by providing electrical and mechanical manipulanda (e.g., Chamove, in prep.; Markowitz and Woodworth, 1978; Murphy, 1976), or appropriate social stimulation (Chamove, 1973), or by attempting to approximate a more natural environment, for example by providing the animals with a deep-litter substrate on floors that were bare (Chamove and Anderson, 1979). The present article reports the results of the three studies concerned with two techniques of enhancing captive conditions for primates. Two studies examined the suitability of woodchips as a deep litter for various primate species. The third study also evaluated the effects of freezing fruit on its distribution and on aggressive behavior during feeding in a macaque group.

Study 1

A previous paper (Chamove and Anderson, 1979) suggested that litter was an effective floor covering for captive macaque groups. The rationale for its use was as follows: If an animal in its natural environment spends a substantial amount of time exhibiting a particular type of behavior, e.g., searching for food, while the animal in captivity is prevented from engaging in similar types of activity, the distortion in the animal's usual pattern of activity might be stress-

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